EVALUATION OF *ANAPLASMA MARGINALE* MAJOR SURFACE PROTEIN 3 (MSP3) AS A DIAGNOSTIC TEST ANTIGEN

By

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This document is dedicated to my beautiful wife Mary, my wonderful children, Arthur and Grace, and to God who we love and serve.

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ABBREVIATIONS

bp	base pair (s)
BSA	bovine serum albumin
°C	degrees Celsius
CD	card agglutination test
CF	complement fixation test
CHEF	clamped homogeneous electric field electrophoresis
CT	capillary tube agglutination test
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fig.	figure (s)
FL	Florida
g	gram (s)
HCl	hydrochloric acid
HRP	horseradish peroxidase
IFA	indirect fluorescent antibody
IIF	indirect immunofluorescence test
IPTG	isopropylthio-β-galactosidase
kbp	kilobase pair (s)
kDa	kilodalton

M	molar
MAb	monoclonal antibody (s)
MAP1	major antigenic protein 1
mg	milligram (s)
min.	minute (s)
ml	milliliter (s)
mM	millimolar
MSP1α	major surface protein 1α
MSP1β	major surface protein 1β
MSP2	major surface protein 2
MSP3	major surface protein 3
N	normal
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram (s)
NP-40	nonidet P-40
PBS	phosphate buffered saline
PCV	packed cell volume
PI	post-infection
PMSF	phenylmethylsufonly fluoride
RFLP	restriction fragment length polymorphism
RIA	radioimmunoassay
rRNA	ribosomal ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate, polyacrylamide gel electrophoresis
sec.	seconds

SI	South Idaho
TBE	tris-borate-EDTA buffer
TE	tris-EDTA buffer
Tris	tris hydroxymethyl aminoethane
TWEEN 20	polyoxyethylene-sorbitan monolaurate
TX	Texas
V	volts
VA	Virginia
w/v	weight/volume
WA	Washington
μg	microgram (s)
μl	microliter (s)

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

EVALUATION OF *ANAPLASMA MARGINALE* MAJOR SURFACE PROTEIN 3 (MSP3) AS A DIAGNOSTIC TEST ANTIGEN

By

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The immunodominant surface protein, MSP3, has been proposed as an antigen suitable for the diagnosis of bovine anaplasmosis. In this study we further characterized MSP3 to examine its potential as a test antigen for the serological detection of carrier cattle. The specificity of MSP3 was evaluated by probing immunoblots of *A. marginale* proteins with immune sera from animals infected with related organisms. Similarly, we used polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblots to evaluate the conservation of MSP3 between 4 different geographic isolates of *A. marginale*. In addition, proteins from a FL isolate were separated by 2-dimensional gel electrophoresis, and immunoblotted with immune sera from cattle infected with one of 4 different geographic isolates of *A. marginale*. Genomic *A. marginale* DNA was digested with restriction endonucleases, transferred to nylon

membranes, and probed with a digoxigenin-labeled, cloned gene of MSP3 (courtesy of Dr. G. Palmer, W.S.U.).

Immunoblots demonstrated cross reactivity between MSP3 and sera from animals infected with *A. ovis, E. risticii,* and *E. ewingii*. Size polymorphism of MSP3 was seen between different geographic isolates of *A. marginale*. Two-dimensional gel electrophoresis revealed at least 3 different antigens migrating at the 86kDa molecular mass, and sera from animals infected with different isolates reacted with different 86kDa antigens. Hybridization studies with a cloned MSP3 gene identified multiple copies of the gene in the genome. These results indicate MSP3 is, 1) cross reactive with *A. ovis* and some *Ehrilichia sp.*, 2) not conserved between different isolates of *A. marginale*, and 3) in at least the FL isolate, MSP3 is actually a group of 3 or more 86kDa proteins with different isoelectric points. These data also suggest *A. marginale* may antigenically vary this immunodominant protein by use of a complex multigene family. The variability of MSP3 between isolates, the multiple 86kDa antigens in the FL isolate, and the multiple copies of the MSP3 gene indicate a single recombinant form of MSP3 may not be a suitable diagnostic test antigen. To be used as a diagnostic test antigen, conserved epitopes between copies of MSP3 genes may need to be identified and tested for reactivity with immune sera.

CHAPTER 1
INTRODUCTION

Anaplasma marginale is an arthropod-borne, rickettsial hemoparasite which invades the red blood cells of cattle causing a clinical disease known as anaplasmosis. This organism is the representative species of the genus *Anaplasma*, in the family Anaplasmataceae, order Rickettsiales (Ristic and Krier, 1974). The genus name, *Anaplasma,* refers to the appearance of being devoid of cytoplasm, and the species name, *marginale,* refers to the location of the organism in the margin of infected erythrocytes. *A. marginale* was originally discovered in cattle experimentally infected with *Babesia bigemina*, and was thought to be a developmental stage of this organism (Smith and Kilborne 1893). However, several years later these marginal inclusions noted in the erythrocytes of cattle infected with *B. bigemina* were conclusively identified as agents belonging to another genus, *Anaplasma* (Theiler 1910).

A. marginale is now known to have a global distribution which includes the United States of America. World-wide economic losses are difficult to calculate, but losses in the U.S. alone are estimated to be over 100 million dollars annually (Goodger et al., 1979; McCallon, 1973). In certain

areas of the U.S., the incidence of infected cattle may be as high as 37% (Maas et al., 1986; McCallon, 1973).

Acute anaplasmosis is usually seen in cattle over 1 year of age and is characterized by a severe hemolytic anemia, resulting in weight loss, abortion, decreased milk production, and often death in infected animals over 3 years of age (Wanduragala & Ristic, 1993). In acute stages, the disease is easily diagnosed by finding organisms on routine blood smear evaluation. However, animals that survive the infection will remain carriers and maintain a low level of parasitemia which cannot be detected microscopically (Richey, 1981; Zaugg et al., 1986). These carrier cattle serve as a perpetual source of infection for susceptible cattle (Swift & Thomas, 1983). Cyclic rickettsemia has been detected and quantitated in carrier cattle using nucleic acid probe hybridization (Eriks et al., 1993; Kieser et al., 1990). These studies demonstrated rickettsemia levels in persistently infected cattle fluctuated at approximately 5 week intervals from a low of 10^4 to a high of 10^7 infected erythrocytes per ml of blood. Although the level of parasitemia was too low to be detected microscopically, uninfected Dermacentor andersoni ticks were able to acquire infection from the cattle at infectivity rates of up to 80% during the higher rickettsemia levels (10^7 infected erythrocytes per ml of blood) (Eriks et al., 1993). Even at extremely low levels of parasitemia 27% of the male ticks became infected. In addition, once ticks acquire the

infection, replication of the organism in the vector allowed easy transmission of the disease with only a few infected ticks regardless of the initial infecting dose. This firmly establishes the important role persistently infected carrier cattle play in the transmission of the disease. In order to reduce economic losses associated with anaplasmosis, control efforts must include an effective way of identifying and decreasing transmission from carrier cattle.

An inexpensive, sensitive, and specific field test for the identification of *A. marginale* infected carriers would have a tremendous impact on limiting the spread and consequently the economic losses associated with this disease. Entire herds could be easily tested and identified carriers removed or treated with oxytetracycline, thus eliminating the source of infection for susceptible cattle. A test such as this would also provide an accurate means of identifying carrier animals being shipped into nonendemic or noninfected areas. Ideally, animals infected with the less virulent species, *Anaplasma centrale*, should not contain antibodies that will cross react with the antigen used in this test. This would be a marked improvement over other serological tests which cannot distinguish between the two infections. In addition, in areas where vaccination with *A. centrale* is used as a means of prevention of *A. marginale*, this test may distinguish vaccinated from infected animals.

The purpose of this study was to evaluate the MSP3 protein of *A. marginale*, and determine if a recombinant form of this protein would be a suitable diagnostic test antigen to detect *A. marginale* infection in carrier cattle.

CHAPTER 2
LITERATURE REVIEW

General Methods of Serologic Diagnosis

Serology is the science of detection of specific antibodies in body fluids, particularly, though not exclusively, serum. There are 3 broad categories of serologic techniques, the primary binding tests, secondary binding tests, and tertiary binding tests. Primary binding tests allow antigen and antibody to combine, and the resulting immune complexes are measured using radioisotopes, fluorescent dye, or enzyme labels. Examples of the primary binding tests are radioimmunoassays, immunofluorescence assays, and enzyme-linked immunosorbent assays. Primary binding tests are the most sensitive of the serologic techniques in terms of ability to detect smaller amounts of specific antibodies (Tizard, 1992).

Radioimmunoassays are widely used primarily because of their extreme sensitivity and ability to detect small amounts of antigen or antibody. In these assays radioactive isotopes such as ^{125}I are used to label antigens or antibodies, and the level of radioactivity is used for quantitation. Disadvantages of this test are the dangers and restrictions

regarding the use of radioactivity, the complexity of the test procedure, and the need for specialized equipment. Because of their extreme sensitivity, these tests are frequently used to measure trace amounts of drugs in urine or other body fluids (Tizard, 1992).

Immunofluorescence assays employ the use of fluorescent dyes such as fluorescein isothiocyanate (FITC) to measure the formation of immune complexes. FITC is easily conjugated to immunoglobulins for detection using dark field microscopy with an ultraviolet light source, or flow cytometry. Specific immunoglobulins may be labeled directly and allowed to react with an antigen as in direct fluorescent antibody tests, or species specific antiglobulins may be labeled with FITC and used to bind to antibody/antigen complexes in indirect fluorescent assays. The indirect tests are usually more sensitive since each antibody molecule bound to the antigen may bind several labeled anti-globulin molecules (Tizard, 1992).

Enzyme-linked immunosorbent assays (ELISA) employ the use of enzymes for the detection of antigen/antibody interactions. The three most commonly used enzymes are alkaline phosphatase, horseradish peroxidase, and β-galactosidase. In these techniques the enzymes are chemically linked to immunoglobulins or anti-globulins. Detection is accomplished by the addition of an enzyme substrate to the reaction,

producing a colored product. The color change may be estimated visually or determined spectrophotometrically.

There are several variations of the ELISA test; the direct ELISA which utilizes enzyme-linked immunoglobulins, the indirect ELISA which uses enzyme-linked anti-globulins, and the competitive ELISA which employs the use of a labeled monoclonal antibody to compete with specific antibodies in the test sample for a single epitope on an antigen molecule. Some ELISAs bind antibody to a solid phase media to capture a particular antigen (antigen capture ELISA), while other techniques bind antigen to the solid phase in order to capture and detect antibody (antibody capture ELISA) (Harlow & Lane, 1988). Various solid phase media may be used such as nitrocellulose membranes or polystyrene microtiter plates, depending on the purpose of the test and the nature of the material to be tested. The extreme versatility of this technique, its excellent sensitivity (comparable to RIA), and its simplicity make it one of the most widely used immunodiagnostic techniques for many viral, bacterial, and parasitic infections. In addition, it does not involve the use of hazardous radioactive isotopes. Because of its extreme sensitivity, specificity may be a problem with these tests, particularly if unpurified test antigens are used to detect specific antibodies in polyclonal sera.

Secondary binding tests measure the interaction of antigen and antibody by in vitro visualization of a secondary event which occurs as a result of immune complex formation. Such events include the precipitation of soluble antigens in solution by immune complex formation, agglutination of particulate antigen on the surface of bacteria or erythrocytes, and the activation of the complement pathway, resulting in cell lysis. Many of these tests require optimal concentration of antigen and antibody to allow visualization, and false results may occur in cases of antibody or antigen excess (Nakamura et al., 1988). As a result of this, and the gross visual detection required for many of these tests, secondary binding tests lack the sensitivity of primary binding assays. Examples of these tests include immunoprecipitation, immunodiffusion, agglutination, and complement fixation.

Tertiary binding tests actually measure the in vivo protective effects specific antibodies may have in an animal. These test measure the biological activity of the antibody to determine their ability to protect a susceptible animal from an infectious agent or neutralize the effects of an antigen. These tests are most useful in experimental trials or in treatment of certain diseases by passive transfer of antibody. They are not practical or suitable for the diagnosis of disease processes.

The suitability of a particular type of immunodiagnostic test depends on many factors such as the use of test (field versus laboratory use), the sensitivity and specificity required, the prevalence of the disease, the number of samples to be tested at any one time, and the speed at which a test must be performed. In general, selection of a diagnostic test often involves a compromise trade off between sensitivity, specificity, and ease of performance. As previously mentioned, the indirect ELISA is a very sensitive test, easy to perform, and may be adapted to field use where multiple samples may be tested simultaneously. It is a primary binding assay and requires no specialized equipment to perform the test or interpret the results. This may prove to be an ideal test for the field detection of *A. marginale* infected carrier cattle. Because of the extreme sensitivity of the indirect ELISA, specificity can be a problem when detecting specific antibodies in a polyclonal sera. To achieve acceptable specificity, great care must be taken in selection and purification of an appropriate test antigen.

Methods for Detection of *A. marginale* Infected Cattle

Currently an effective vaccine against *A. marginale* is not available, and the inability of present serologic tests to accurately detect persistently infected, carrier cattle severely compromises efforts to establish disease free herds and reduce economic losses through testing, isolation, and

treatment (Luther et al., 1980; Richey, 1981). Several types of serological tests have been described for the diagnosis of *A. marginale*, including complement fixation (CF), capillary tube agglutination (CT), card agglutination (CD), indirect immunofluorescence (IIF), and ELISA (Amerault & Roby, 1968; Barry et al., 1986; Duzgun et al., 1988; Gonzalez et al., 1978; Kuttler, 1981). Error rate with these tests is high, primarily because antigens used in these tests are a crude mixture of *A. marginale* and erythrocyte material (Kocan et al., 1978; Kocan et al., 1978). False positives as high as 20% are seen due to poor specificity of test antigen (Amerault & Roby, 1968; Amerault et al., 1973; Barry et al., 1986; Duzgun et al., 1988; Gonzalez et al., 1978; Luther et al., 1980; Todorovic et al., 1977). Poor sensitivity of these tests results in false negatives as high as 21% (Amerault et al., 1973; Barry et al., 1986; Goff et al., 1990; Gonzalez et al., 1978; Luther et al., 1980; Maas et al., 1986). Current technology, which was not available when previous tests were developed, allows us to economically produce a sensitive and specific ELISA test for the rapid field diagnosis of *A. marginale* using purified, recombinant, *A. marginale* protein.

As previously mentioned, several types of tests have been developed for the diagnosis of anaplasmosis. The tests which have received most attention have been the rapid card agglutination test (CA), the complement fixation test (CF),

indirect immunofluorescence test (IIF), the radioimmunoassay (RIA), and various enzyme-linked immunosorbent assays (ELISA).

The Rapid Card Agglutination Test (CA)

The CA is one of the most widely used in the field because it is easy to run, requires minimal equipment, and uses unheated sera or heparinized plasma (Amerault & Roby, 1968). Being a secondary binding test, this assay lacks the sensitivity of primary binding assays. Initial studies using the CA test reported 100% sensitivity in detecting carrier cattle and 86% sensitivity in detecting all known infected cattle (Amerault & Roby, 1968). However, the number of positive cattle tested was small (22), and subsequent studies comparing sensitivity and specificity of the CA test with other serological tests indicates sensitivity to be 84% (Gonzalez et al., 1978). Yet another study indicated the CA test was able to detect only 6 of 9 (66.6%) known carrier cattle (Luther et al., 1980). In addition, 89.3% of noninfected, *A. marginale* vaccinated cattle had positive reactions with the CA test for up to 15 months after vaccination (Luther et al., 1980).

Specificity of the CA test was initially reported to be 100% with none of the 24 normal cattle sera testing positive (Amerault & Roby, 1968). This test uses a crude preparation of *A. marginale* and erythrocyte antigen, and problems of specificity are likely to be encountered. Nonspecific

agglutination did occur when fresh serum was used; however, this problem did not occur when using heparinized plasma. Additional studies involving large numbers of serum samples (380) established 98% specificity for the CA test (Gonzalez et al., 1978). However, none of these studies investigated the potential for cross reactivity with sera from cattle infected with other hemoparasites. Current technology, which was not available when previous tests were developed, allows the phylogenetic relatedness of various species and genera to be determined. Using 16s rRNA sequence analysis investigators have determined *A. marginale* to be closely related to other *Anaplasma sp.*, *Ehrlichia sp.*, and *Cowdria ruminantium* (Dame et al., 1992; Van Vliet et al., 1992). To accurately determine the specificity of a test antigen, it is essential to test the ability of the antigen to distinguish between infections of related organisms. This was not done for any of the antigens currently used in serologic diagnosis of *A. marginale*. Therefore estimations of specificity for these tests are unreliable.

Although the CA test is well adapted for field use, its poor sensitivity (probably because it is a secondary binding test) and its questionable specificity (because of its use of a crude test antigen preparation) make it an undesirable test for the rapid field identification of carrier cattle.

The Complement Fixation Test (CF)

The CF is another widely used test for the detection of antibodies to *A. marginale* in infected cattle. This test relies on the ability of *A. marginale* antibodies in the serum to fix complement and lyse target cells on which the antigen is associated (Kuttler & Winward, 1984). The antigen used in this test is again a crude mixture of *A. marginale* and erythrocyte proteins, and since it is a secondary binding test, sensitivity is not as high as with primary binding assays. Sensitivity of this assay is reported to be low, varying from 10% to 79% (Gonzalez et al., 1978; Goff et al., 1990). In addition, it has been shown that CF antibodies decline rapidly after natural infections with *A. marginale* (Todorovic et al., 1977). CF antibodies decreased to very low levels (1:10) as soon as 10 weeks post-infection (PI), and below the sensitivity level of the test (1:5) by 14 weeks PI (Gonzalez et al., 1978). This may be because CF relies on the presence of IgM antibodies, which are better able to fix complement than IgG. IgM levels are elevated in acute infections, but may decline after acute episodes subside. These results would indicate this test is unreliable in detecting carrier cattle. In another study, 65 cattle from a known *A. marginale* infected herd were tested by CF and DNA probe hybridization (Goff et al., 1990). Stained blood smears prepared from these cattle detected no organisms. However, 64 of the 65 cattle blood samples tested positive by DNA probe

hybridization and 60 of the 65 sera tested positive by IIF, while only 5 of the 65 tested positive by CF.

The specificity of the CF test in detecting non-infected cattle is very high, up to 100% (Gonzalez et al., 1978). This is not surprising given the low test sensitivity. However, the study again looked at specificity involving the detection of CF antibodies in normal, non-infected cattle, not cattle which may be infected with other closely related hemoparasites. Therefore, cross reactivity with cattle infected with other rickettsial or hematoprotozoal agents may occur. In addition, as with the CA test, the CF test could not distinguish between infected animals and animals vaccinated with the killed *A. marginale* vaccine (Luther et al., 1980).

The CF test is a tedious test to perform and requires considerable technical skill and knowledge. Therefore, the complexity of the test and its poor sensitivity, particularly in detecting infected carriers makes it a poor choice for the field identification of infected animals.

The Indirect Immunofluorescence Test (IIF)

The indirect immunofluorescence test (IIF), being a primary binding assay, is much more sensitive than the CA or CF tests. This test uses *A. marginale* infected erythrocytes fixed to a microscope slide and permits reaction to them using a patient's serum. Antibodies to *A. marginale* on infected

erythrocytes are then visualized using a fluorescein-conjugated anti-bovine, rabbit immunoglobulin (Gonzalez et al., 1978). Sensitivity in detecting subclinically infected animals is reported to be 97% with lower limits of sensitivity not being reached by 18 weeks PI. In another study involving 64 cattle naturally infected with *A. marginale* and confirmed by DNA probe hybridization, 94% of cattle were positively identified by IIF (Goff et al., 1990). In this study circulating organisms were not seen in stained blood smears, but length of infection was undetermined.

Although sensitivity with IIF is much improved over CA and CF tests, the specificity is somewhat reduced with 10% of normal, non-infected cattle testing positive by IIF (Gonzalez et al., 1978). In addition, cross reactivity in cattle infected with related organisms may also present a problem. The sensitivity of this test is satisfactory, however, its questionable specificity, labor intensive nature, and need for specialized reagents and equipment make it undesirable as a field test for routine diagnosis and identification of *A. marginale* infected carriers.

The Radioimmunoassay (RIA)

Recently a radioimmunoassay (RIA) was developed for the detection of *A. marginale* antibodies in sera of infected cattle (Schuntner & Leatch, 1988). This test initially demonstrated high specificity and sensitivity (98.8% for each)

when testing a large number of *A. marginale* infected cattle, normal cattle, and cattle infected with *B. bigemina, B. bovis*, and *Theileria orientalis*. This test utilizes a crude *A. marginale* antigen preparation isolated from infected erythrocytes. Reactants are identified using ^{125}I-labeled, anti-bovine IgG, rabbit immunoglobulin and an automated gamma counter. As expected, sensitivity with this primary binding assay is high; however, substantial numbers of false positives (up to 37%) occurred unless sera was pre-absorbed with normal bovine erythrocytes and sonicated *B. bovis* antigen. Controls for cross reactivity using pre-absorption should not be necessary if purified antigens are used, even with tests as sensitive as RIAs.

Even though the RIA is highly sensitive and specific, it is too labor intensive to be used as a practical field test where large numbers of samples need to be processed. In addition, the use of radioactive material and the need for specialized equipment limits its use to reference or research laboratories.

Enzyme-Linked Immunosorbent Assays (ELISA)

Several ELISAs for measuring antibody to *A. marginale* have been developed over recent years (Barry et al., 1986; Duzgun et al., 1988; Montenegro-James et al., 1990; Nakamura et al., 1988; Trueblood et al., 1991; Winkler et al., 1987). Because these are primary binding assays, the sensitivity of

most of these tests are good. However, none of these tests use a single, purified *A. marginale* antigen, all use a crude mixture of *A. marginale* antigens prepared from initial bodies. Therefore, test specificity has been a problem, as is frequently encountered using a mixture of proteins as a test antigen in a highly sensitive technique.

Barry et al., 1986 developed one of the early ELISAs for diagnosis of *A. marginale* infection in cattle. Using a crude mixture of *A. marginale* initial bodies and cell membranes from ghost RBC's as a test antigen, the test reportedly was able to accurately distinguish cattle free from infection from recently infected animals (up to 8 months duration). However, no measures were taken to insure the positive or negative status of most of the animals tested. Also, test accuracy for identifying carrier cattle was questionable having identified only 2 of 3 cattle infected > 3 years. In addition, the test appeared to be fairly insensitive, necessitating the testing of sera at a dilution of 1:100. Using concentrated serum for testing may cause a problem with test specificity and 14.6% of cattle inoculated twice with *B. bovis*-infected erythrocytes were positive for *A. marginale* using this ELISA. Cross-reactions were attributed to antibodies produced against RBC antigens in the inoculum. Experiments investigating cross reactivity with other closely related rickettsial or hematoprotozoal agents were not performed.

Another diagnostic test utilized the CF test antigen in particulate and SDS-solubilized form as a test antigen in an ELISA (Winkler et al., 1987). This study indicated SDS-solubilization of the proteins decreased background reactivity in the test and increased test sensitivity in detecting positive reactants. Complete correlation between the ELISA and CF test was found when using solubilized test antigen and CF test positive and negative reference sera. In testing other infected cattle, false negatives were not observed; however, cross reactivity ranged from 50% to 80% when testing sera from cattle infected with or immunized against different infectious agents.

Nakamura et al., 1988, developed an ELISA test antigen by nitrogen decompression of infected cells to isolate initial bodies, and solubilized them in triton X-100. Test specificity and sensitivity was determined by comparison of results with the CF test. Specificity of this ELISA was satisfactory having 100% agreement with sera tested negative by CF and no cross reactivity with serum from animals infected with *Babesia sp., Theileria sp.*, or *Eperythrozoon sp.* Cross reactivity was detected with *A. centrale*. Cross reactivity with other rickettsial agents such as *Ehrlichia sp.* or *Cowdria ruminantium* was not determined. Sensitivity of this test was not adequately determined since the results were compared to a test (CF test) which itself has poor sensitivity, particularly in detecting carrier cattle. No effort was made

to test the sensitivity of this ELISA in detecting infected carriers shown to be positive by other proven sensitive tests such as IIF or calf inoculations.

Another ELISA used a 2 antigen technique to enhance test specificity; a negative antigen prepared from a cow prior to infection, and a positive antigen derived from *A. marginale* infected cells (Duzgun et al., 1988). Reactants were identified using net absorbance values obtained by subtracting the absorbance value of sera with negative antigen from the absorbance value of sera with positive antigen. Specificity of this test was good with only 3% of negative sera, 2% of sera from animals infected with *B. bovis*, and 4% of sera from animals infected with *B. bigemina* giving positive results. Other related rickettsial agents were not tested. Sensitivity also appeared to be good with no false negatives noted in 100 animals confirmed negative by IIF or calf inoculations. A small number of infected cattle had positive ELISAs for up to 3 years later. This test provided the best sensitivity and specificity thus far, and was the first ELISA that demonstrated the ability to detect long term carrier cattle. However, the crude nature of the test antigen necessitates the use of the 2 antigen system which is more cumbersome, complex, and time consuming. If this 2 antigen system had not been used, 37% of the negative sera would have given false positive results. In addition, the 2 antigen system requires the use of a spectrophotometer to identify reactants. This does not

allow for eventual visual identification as would be needed for rapid field diagnosis.

Direct visualization of positive reactants was accomplished using a Dot-ELISA (Montenegro-James et al., 1990). In this test whole initial body preparations were solubilized in SDS and dotted on to nitrocellulose disks. Test sera were reacted with the antigen and antigen/antibody complexes were visualized with alkaline phosphatase-conjugated protein A. Test specificity was good (95%) and cross reactivity to *Babesia* or *Trypanosome vivax* was not observed. However, other rickettsial agents or related hemoparasites were not tested. Test specificity was increased by using protein A-conjugated alkaline phosphatase versus an anti-globulin-conjugated molecule. This reduced nonspecific binding of antibodies to the nitrocellulose disk. However, the use of whole initial bodies isolated from infected erythrocytes still lends itself to false positive reactions.

Test sensitivity with the Dot-ELISA was fair (92.9%) with 19 false negatives out of 269 true positives (Montenegro-James et al., 1990). In addition, no effort was made to determine the ability of this test to detect chronically infected, long term carrier cattle. A good diagnostic test for identification of infected cattle for importation or herd management should have a sensitivity of at least 95% or higher. Therefore, while this test is easy and convenient to

perform, its lack of sensitivity and questionable specificity are certainly areas of needed improvement.

Recently, an antigen capture ELISA was developed for the detection of *A. marginale* infection (Trueblood et al., 1991). This ELISA utilizes 2 monoclonal antibodies (MAb) which recognize 2 different epitopes on the *A. marginale* surface protein MSP-1α. In this test one MAb is bound to a polystyrene microtiter plate, antigen (ie. infected whole blood) is incubated with this monoclonal, and a second monoclonal conjugated to horseradish peroxidase is added to the reaction mixture for visualization of any captured antigen. This assay was sensitive enough to detect infected animals with parasitemias of <1.0%, however, it is not sensitive enough to detect carrier cattle which have parasitemias as low as 10^4 infected cells per ml of blood (0.0001%) (Eriks et al., 1993). Thus, although the detection of *A. marginale* antigen in the blood of infected cattle would be the most specific way to determine infection, the sensitivity of most primary binding assays will not likely be sufficient to detect the low level of parasitemia encountered in carrier cattle.

Nucleic Acid Probe Hybridization

One test which has shown excellent results in the ability to detect *A. marginale* organisms in infected cattle, even at a level of sensitivity sufficient to identify chronically

infected carriers, is a nucleic acid probe derived from a fragment within the gene coding for an *A. marginale* surface protein (Goff et al., 1988; Eriks et al., 1989). This probe can detect infected animals with parasitemias as low as 0.000025% (Eriks et al., 1989). Sensitivity and specificity of the test surpasses all previously developed serological tests, including the IIF (Goff et al., 1990). Although this test is too complex to be useful for routine field diagnosis, it has tremendous potential for identification of true carriers which in the past was done by subinocculation of splenectomized calves. This could be useful for the identification of cattle for reference sera, establishing the effects treatment with tetracycline has on carrier status, or identification of infected ticks. Considering the alternative of calf inoculation, a nucleic acid probe is a much more convenient and economical tool when accurate identification of infection is essential.

Previous Experiments

The ideal test antigen to detect *A. marginale* infected carriers must be antigenic enough to have antibodies present in sera of cattle during all stages of infection. It must be species specific so as not to cross react with sera from animals infected with related organisms, and it must be conserved between isolates of *A. marginale*, enough to be recognized by sera from cattle infected with various

geographic isolates. An antigen which shows promise for meeting the above requirements is the 86kDa surface protein of *A. marginale*, MSP3.

In previous experiments, the most antigenic surface proteins of *A. marginale* were identified by immunoprecipitation of radiolabeled initial body proteins from a Florida (FL) isolate of *A. marginale* with immune sera from infected cattle (Palmer et al., 1986). The FL isolate of *A. marginale* was used for antigen isolation because it has been found by adsorption studies to contain antigens common to both morphologic types of *A. marginale*, the tailed and non-tailed forms (Goff and Winward, 1985; Kreier and Ristic, 1963). An 86kDa protein, MSP3, was identified as the most immunodominant in all stages of infection from as early as 30 days post-infection (PI) to 255 days PI. Similar reactivity was observed when initial body preparations were immunoprecipitated with immune sera from cattle infected with either of 3 different isolates of *A. marginale*, FL, Virginia (VA), and Texas (TX) isolates (Palmer et al., 1986). This suggested MSP3 from a FL strain was conserved enough to be recognized by immune sera from animals infected with different isolates. However, the conservation of MSP3 was not firmly established since these studies were performed using single dimensional gel electrophoresis.

These experiments identified for the first time antigenic surface proteins of *A. marginale* which could be investigated

for potential use as a diagnostic test antigen or vaccine candidate. Use of a single antigen in a diagnostic test could markedly increase test specificity over currently available tests. Since MSP3 was the most immunodominant protein, further experiments were done to determine its potential as a diagnostic test antigen. Monoclonal antibodies to MSP3 were produced and used in a sepharose bead affinity column to isolate purified MSP3 from FL isolate initial bodies (McGuire et al., 1991). Affinity purified MSP3 was injected into rabbits and rabbit-anti-MSP3 immune serum was used on dot blots to identify epitopes of MSP3 in at least 8 different geographic isolates of *A. marginale* (Mcguire et al., 1991). Using immune sera from infected cattle, purified MSP3 accurately identified long term carriers for up to 5 years PI. Immune sera from cattle infected with *B. bovis*, *B. bigemina*, or an unidentified rickettsial agent did not cross react with purified MSP3 (McGuire et al., 1991). However, sera from animals infected with organism now known to be phylogenetically related to *A. marginale* were not tested in these experiments.

Since MSP3 fulfilled many of the criteria for a good diagnostic test antigen, attempts were made to clone the MSP3 gene and produce a recombinant protein which might be used in an ELISA test. A genomic library made from a FL isolate of *A. marginale* DNA was screened using a pool of anti-MSP3 MAbs. Three clones of the MSP3 gene were identified and sequenced.

However, proteins expressed by these clones were inconsistent in their reactivity with immune cattle sera. In addition, in depletion experiments, anti-MSP3 MAbs were reacted with FL *A. marginale* initial bodies. The initial bodies still contained an 86 kDa antigen when reacted with immune cattle sera.

The MSP3 antigen of *A. marginale* appears to be a strong candidate as a diagnostic test antigen to detect infected carrier cattle. However, these previous experiments propose questions which must be answered to confirm this hypothesis. Questions must be addressed regarding the specificity of this protein in detecting *A. marginale* infection, the conservation of the MSP3 protein between various geographic isolates of *A. marginale*, and explanations for inconsistent reactivity of the 3 MSP3 clones. The purpose of our investigation is to further characterize the MSP3 protein and determine if it is a suitable candidate for a diagnostic test antigen to detect *A. marginale* infected, carrier cattle.

CHAPTER 3
MATERIALS AND METHODS

Anaplasma marginale Strains

Four isolates of *A. marginale* were used in this study, FL, VA, South Idaho (SI), and Washington (WA). These isolates are designated by their original location of isolation (McGuire et al., 1984). Isolates were stored in liquid nitrogen as cryopreserved stabilates (Love, 1972) before being used to infect splenectomized calves. Thawed stabilate (20ml) from each isolate was injected intramuscularly into 6-month-old, male Holstein calves. Calves were monitored daily for percent parasitemia by blood smear evaluation, and packed cell volume (PCV). Infected, whole blood was collected in EDTA from calves during periods of peak parasitemias (FL=70%, VA=36%, SI=42%, and WA=40%), centrifuged at 10,000 x g for 15 min., and the serum and buffy coat was removed. Packed erythrocytes were washed 3 times in phosphate buffered saline (PBS) (0.14M NaCl, 2.68 mM KCl, 8.26 mM K_2HPO_4, pH 7.4), resuspended to a PCV of 50%, and stored at -70^0C.

A. marginale strains used in clamped homogeneous electric field electrophoresis (CHEF) studies were prepared as previously described (Alleman et al., 1993). Briefly, whole

blood from cattle infected with the previously mentioned isolates was collected with sodium heparin used as an anticoagulant and washed 3 times in PBS. Erythrocytes were separated from bovine leukocytes by passing washed blood over an α-cellulose/microcrystalline cellulose column (Sigmacell type 50, Sigma Chemical Co., St. Louis, MO) as described by Beutler (Beutler, 1984), except that 1% bovine serum albumin (BSA) was included in the wash buffer. Washed cells were resuspended to a concentration of 5.0×10^6 cells/μl, or approximately 35% packed cell volume (PCV). Blood films were prepared from isolated erythrocytes for microscopic detection of bovine leukocyte contamination and level of parasitemia.

Intact erythrocytes were embedded in 0.7% agarose by mixing 1 part erythrocyte suspension with 2 parts of 1% FMC InCert Agarose (FMC Bioproducts, Rockland, ME) in PBS (0.14M NaCl, 2.68 mM KCl, 8.26 mM K_2HPO_4, pH 7.4), 0.125 M EDTA. Mixtures of blood and agarose were kept at 37°C while pipetting into molds. Molds containing plugs were placed on ice to set. Plugs were incubated in 0.5 M EDTA (pH 9.5), 1% N-lauroylsarcosine, and 2 mg/ml Proteinase K for 48 hours at 37°C, then stored at 4°C in fresh Proteinase K solution.

Initial Body Preparation

A. marginale initial bodies were isolated from infected erythrocytes as previously described (Palmer and McGuire, 1984). Briefly, frozen blood was quick-thawed at 37°C and

washed 5 times in PBS with each centrifugation at 16,000 x g for 25 min. at 4°C. After each centrifugation, an upper layer containing both leukocytes and erythrocytes was removed. The pellets were resuspended in PBS, sonicated for 2 min. on ice at 50 W and centrifuged as before. Pelleted material was again resuspended in PBS, and sonicated for 30 sec. on ice at 50 W and centrifuged a final time. Intact initial bodies were visualized by Wright-Giemsa stain. The pellets of initial bodies were resuspended in equal volumes of PBS for use in SDS-PAGE.

Initial bodies used in 2-D gel electrophoresis were resuspended in equal volumes of lysis buffer containing 9.5 M urea, 2% nonidet P-40, 1.6% Ampholyte 5/7 (Bio-Lyte 5/7, Bio-Rad Laboratories, Richmond, CA), 0.4% ampholyte 3/10 (Bio-Lyte 3/7, Bio-Rad Laboratories, Richmond, CA), and 5.0% β-mercaptoethanol.

Protein concentrations were determined spectrophotometrically using the Micro BCA Protein Assay (Pierce, Rockford, Illinois). Initial body preparations were stored in small aliquots at -70°C. *Anaplasma centrale* initial bodies were also prepared as described above.

Babesia bovis and *Babesia bigemina* Antigen Preparation

Babesia bovis and *B. bigemina* antigens were prepared from organisms maintained in microaerophilic stationary phase culture as previously described (Levy and Ristic, 1980).

Briefly, infected erythrocytes were centrifuged at 10,000 x g and the supernate was removed. Packed cells were resuspended to 20 times the volume in 10 mM sodium phosphate. The solution was centrifuged and the supernate was removed. The pellets were then resuspended in an equal volume of 10 mM sodium phosphate.

Antisera used for Immunoblots

Six Holstein calves were infected with blood stabilate containing a FL isolate of *A. marginale*. Two other Holstein calves were each infected with either a VA or a SI isolate. Antisera from all calves were collected at 50 and 70 days PI. Antiserum from a cow experimentally infected with a WA isolate, rabbit-anti-MSP3 polyclonal sera (RB-955), an anti-MSP3 monoclonal antibody (MAb) (AMG75C2), and an anti-MSP2 MAb (ANAF19E2) were supplied to us courtesy of Travis McGuire and Guy Palmer (Washington State University, Pullman). The reactivities of the MAbs (McGuire et al., 1984; McGuire et al., 1991; Palmer et al., 1988; Palmer et al., 1994) and the rabbit-anti-MSP3 polyclonal serum (McGuire et al., 1991) have been previously described. Sera obtained from calves prior to infection with *A. marginale*, and a MAb specific for the variable surface glycoprotein of *Trypanosoma brucei* (TRYP1E1), were used as negative controls in immunoblot experiments.

A. centrale and *A. ovis* antisera with indirect fluorescent antibody (IFA) titers of 1:4,000 were supplied

courtesy of Susan Oberle (The Salk Institute, San Diego, CA). Hyperimmune sera from cattle infected experimentally with *B. bovis* and *B. bigemina* were supplied by David Allred (University of Florida, Gainesville). Sera from cattle experimentally infected with *Cowdria ruminantium* were supplied by Michael Bowie (University of Florida, Gainesville). Equine sera from animals infected with *Ehrlichia equi* and *Ehrlichia risticii* had IFA titers of 1/1,600, and were obtained from Ibulaimu Kakoma (University of Illinois, Urbana). Serum from a dog with canine ehrlichiosis was obtained from Rose Raskin (University of Florida, Gainesville). This dog was infected with *E. ewingii*, but serum from this animal had an IFA titer of 1/160 for *Ehrlichia canis*, and 1/64 for *E. chaffeensis*.

SDS-PAGE

Initial body preparations containing 6.0 - 10.0 μg of protein were solubilized in one half their volume of a 3x sample buffer containing 0.1 M Tris pH 6.8, 5% SDS (w/v), 50% glycerol, 7.5% β-mercaptoethanol, and 0.00125% bromophenol blue, and heat denatured at 100^{0}C for 3 min. Proteins were electrophoresed on 7.5% to 17.5% (w/v) gradient polyacrylamide gels. Gels were fixed in 25 mM Tris, 191.8 mM Glycine and 20% Methanol, and electrophoretically transferred to nitrocellulose (Hybond ECL, Amersham International plc, Buckinghamshire, England).

Two-Dimensional Gel Electrophoresis

Isoelectric focusing gels were prepared per manufacturer's instructions (Protean II Slab Cell Instruction manual, Bio-Rad Laboratories, Richmond, CA). Briefly, an acrylamide\N,N'-methylene-bis-acrylamide solution containing 9.5 M urea, 2.0% nonidet P-40(v/v), 4.1% acrylamide/bis (30.8% T/2.6% C), 10 mM (3-[(3-cholamidopropyl)dimethylammonio]-1propanesulfonate, CHAPS), and 5.8% Bio-Lyte 5/7 (v/v) was polymerized in glass tubing 3 mm x 140 mm. Initial body preparations were incubated for 2 hours at room temperature in 4 times their volume of the previously described lysis buffer and 5 times their volume of sample buffer containing 9.5 M urea, 2.0% Triton X-100, 5% β-mercaptoethanol, 1.6% Bio-Lyte 5/7, and 0.4% Bio-Lyte 3/10. The solution was then centrifuged at 100,000 x g for 2 hrs. at 25^{0}C. A volume of the supernatant containing 20μg of protein was loaded onto each tube gel and overlayed with 50μl of overlay buffer containing 9.5 M urea, 0.8% Bio-Lyte 5/7, 0.2% Bio-Lyte 3/10, and 0.0025% bromophenol blue. Tube gels were electrophoresed at 400 volts for 16 hrs. and 800 volts for 2 hrs. using the BRL model V16 vertical gel electrophoresis system (Bethesda Research Laboratories, Gaithersburg, MD). Tube gels were extruded from the glass tubes and equilibrated for 5 min. in buffer containing 0.0625 M Tris-HCl, pH 6.8, 10.0% glycerol, 2.0% SDS (w/v), 5.0% β-mercaptoethanol, and 0.00125%

bromophenol blue. Focused proteins were then electrophoresed on 7.5% to 17.5% (w/v) gradient polyacrylamide gels and treated and transferred to nitrocellulose membranes as described above.

Immunoblots with Antisera

Nitrocellulose membranes containing transferred proteins were blocked with 5% milk (w/v) in PBS with 0.25% polyoxyethylene-sorbitan monolaurate (Tween 20) to inhibit non-specific binding of primary and secondary antibodies. The membranes were washed with 1% milk (w/v) in PBS with 0.25% Tween 20, and probed with antisera from animals infected with one of the following organisms; *A. marginale*, *A. centrale*, *A. ovis*, *B. bovis*, *B. bigemina*, *C. ruminantium*, *E. equi*, *E. risticii*, or *E. ewingii*. Normal sera from respective uninfected species were used as negative controls. Serum dilutions (in PBS with 1% milk and 0.25% Tween 20) of 1/100 or greater were used. Rabbit-anti-MSP3 polyclonal sera was used at a dilution of 1/5,000. Normal rabbit sera was used at the same dilution as a negative control. Anti-MSP3, anti-MSP2, and negative control anti-trypanosome MAbs were used in concentrations of 5 μg/ml. The membranes were again washed with 1% milk (w/v) in PBS with 0.25% Tween 20 and probed with either species-specific anti-IgG-Horseradish peroxidase (HRP) conjugated antibody at a dilution of 1/2,000 (Sigma Immuno Chemicals, St. Louis, MO), or HRP-conjugated Protein G at a

dilution of 1/15,000 (Sigma Immuno Chemicals, St. Louis, MO). Membranes were processed for enhanced chemiluminescence (ECL) with detection reagents containing luminol as a substrate (ECL Western Blotting detection reagents), (Amersham International, plc, Buckinghamshire, England). The membranes were exposed to Hyperfilm - MP (Amersham International, plc, Buckinghamshire, England) to visualize bound antibody.

Purification of *A. marginale* Genomic DNA

Florida, South Idaho, and Virginia isolates of *A. marginale* genomic DNA used in hybridization studies were purified by phenol/chloroform extraction and ethanol precipitation as previously described (Barbet et al., 1987; Barbet and Allred, 1991). *A. marginale* genomic DNA from FL, SI and VA isolates used in CHEF studies was prepared in agarose plugs as previously described above.

MSP3 Clones

Three clones of the MSP3 gene, MSP3-11, MSP3-12, and MSP3-19 were cloned, sequenced, and supplied to us courtesy of T. McGuire, G. Palmer, and T. McElwain (Washington State University, Pullman). These clones were prepared from a genomic library composed of mechanically sheared *A. marginale* DNA, ligated with *Eco* RI adaptors, and inserted into pBluescript SK(-) plasmids (Stratagene, Lajolla, CA). Clones were identified by screening the library with anti-MSP3 MAbs

(AMG75C2, AMG76B1, AMG43/19, & AMG43/23), one of which, AMG75C2, has been previously described (McGuire et al., 1991). One of these genes, MSP3-12, contains the N-terminus and 633 bp upstream to the open reading frame (Fig. 1). The C-terminus is not present in this clone. The remaining 2 clones, MSP3-11 and MSP3-19, are missing the N-terminal sequence of the open reading frame (Fig. 1). They both contain the C-terminal end of the gene as well as 1,473 bp and 2,480 bp respectively, downstream to the open reading frame.

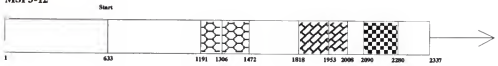
Verification of Recombinant MSP3

To verify a cloned MSP3 gene represented a gene which produced one or more of the 86 kDa antigens seen on 2-D immunoblots, *E. coli* cells (Epicurian Coli XL1-Blue) (Stratagene, Lajolla, CA) were transformed with pBluescript containing each of the 3 MSP3 genes. This was done according to manufacturer's instructions. *E. coli* cells were transformed with nonrecombinant pBluescript as a negative control. Transformed cells were plated on Luria agar containing 50 μg/ml of ampicillin. Transformants were selected by blue/white screening and selected colonies of each transformant were grown overnight in Luria broth containing 50 μg/ml of ampicillin. Transformed *E. coli* cells were suspended in PBS, lysed in one half their volume of a 3x sample buffer containing 0.1 M Tris pH 6.8, 5% SDS (w/v), 50% glycerol, 7.5% β-mercaptoethanol, and 0.00125% bromophenol blue, and heat

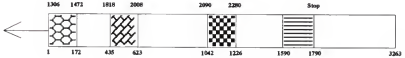
Fig. 1 Diagram of MSP3 clones.
A schematic representation of clones MSP3-11, MSP3-12, and MSP3-19. Homologous regions are indicated by like shaded areas. Nucleotide numbers are indicated on the bottom. Top numbers of clones MSP3-11 and MSP3-19 indicate corresponding nucleotides in clone MSP3-12.

MSP3 CLONES

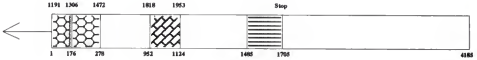
MSP3-12



MSP3-11



MSP3-19



denatured at 100^{0}C for 3 min. Bacterial lysates or similarly prepared FL *A. marginale* lysates were separated by SDS-PAGE and transferred to nitrocellulose as previously described above. Membranes were reacted with anti-MSP3 MAb AMG75C2 or immune sera from animals infected with a FL or VA isolate of *A. marginale* as described above. Nonimmune cattle sera and an anti-*T. brucei* MAb were used as negative controls. Antigen antibody reactions were visualized by ECL with detection reagents containing luminol as a substrate (ECL Western Blotting detection reagents), (Amersham International, plc, Buckinghamshire, England). The membranes were exposed to Hyperfilm - MP (Amersham International, plc, Buckinghamshire, England) to visualize bound antibody.

Digoxigenin Labeling of pBluescript MSP3-12

Empty pBluescript DNA and pBluescript MSP3-12 DNA were grown in previously prepared transformants. Plasmid DNA was isolated from bacterial DNA by ion exchange chromatography using the QIAGEN Plasmid Midi Kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. The purified plasmid DNA was precipitated in ethanol, dried, and dissolved in TE (10 mM Tris, pH 7.5, 1 mM EDTA).

A digoxigenin-labeled probe of pBluescript MSP3-12 was prepared by digestion of 5 μg of pBluescript MSP3-12 with *Eco* RI according to manufacturer's instructions (Boehringer Mannheim Corp., Indianapolis, IN). Empty pBluescript was

digested identically for size comparison of digested plasmids. The 2.3 kbp insert of the MSP3-12 gene, which was previously inserted using *Eco* RI adaptors, was separated from plasmid DNA by electrophoresis on a 1% agarose gel in Tris - Borate - EDTA buffer (TBE) (45 mM Tris, 45 mM Boric acid, 1 mM EDTA). The gel was stained with 0.5 μg/ml ethidium bromide for 20 min. and photographed. The 2.3 kbp band representing clone MSP3-12 was cut from the gel and DNA was extracted from the agarose plug by ion exchange chromatography using QIAquick Gel Extraction Kit (Qiagen Inc., Chatsworth, CA).

A probe was made by random prime labeling 200 ng of MSP3-12 DNA with digoxigenin using the Genius System Nonradioactive DNA Labeling Kit according to the manufacturer's instructions (Boehringer Mannheim Corp., Indianapolis, IN).

Representation of pBluescript MSP3-12 in the *A. marginale* Genome

Restriction Enzyme Digestion

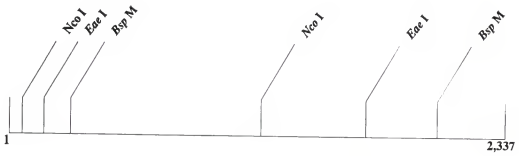
To verify the cloned pBluescript MSP3 was an accurate representation of genomic MSP3, multiple restriction sites of pBluescript MSP3-12 and genomic *A. marginale* DNA from a FL isolate were compared using restriction enzymes which cut within the MSP3 gene. Restriction enzymes *Nco* I (Boehringer Mannheim Corp., Indianapolis, IN), *Bsp* M (New England BioLabs, Beverly, MA) and *Eae* I (New England BioLabs, Beverly, MA), were chosen because they produced large fragments in different

areas of the MSP3 gene (Fig. 2). Digestions of genomic *A. marginale* DNA (1.0 μg) and pBluescript MSP3-12 (0.1 μg) were performed according to the manufacturer's specifications. Digested genomic and plasmid DNA were separated by gel electrophoresis on 1% agarose gels containing 0.1 μg ethidium bromide and photographed.

Southern Blots

Prior to transfer, the gel was incubated at room temperature for 30 min. in 0.4 N NaOH, 0.6 M NaCl then 30 min. in 1.5 M NaCl, 0.5 M tris HCL, pH 7.5. Digested DNA was then transferred to a positively charged, molecular biology nylon membrane (Boehringer Mannheim Corp., Indianapolis, IN) by capillary diffusion using 10x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The filter was washed for 30 sec. in 0.4 N NaOH, then in 0.2 M Tris HCl, pH 7.5, 2X SSC for 2 min. After air drying, the DNA was cross linked by ultraviolet radiation and incubated for 3 hours at 65°C in prehybridization solution containing 6x SSC, 0.5% SDS (w/v) - 200 μg/ml herring sperm DNA, 5x Denhart's [1x Denhart's is 0.02% Ficoll (w/v), 0.02% polyvinylpyrrolidone (w/v), 0.02% BSA (w/v)]. The digoxigenin-labeled MSP3-12 probe was added to fresh prehybridization solution at a concentration of 15 ng/ml, and the filter was hybridized overnight at 65°C. Bound probe was detected by enhanced chemiluminescence using alkaline phosphatase conjugated, anti-digoxigenin IgG

Fig. 2 Representation of clone MSP3-12 in the *A. marginale* genome. Diagram of clone MSP3-12 indicating the restriction enzyme sites and the resulting fragments used to map MSP3-12 to the genome. Nucleotide cleavage site for each restriction enzyme is illustrated diagrammatically (top) and by nucleotide number(bottom). Size of resulting fragments in base pairs is also provided.



Restriction Enzyme	Cleavage site in pBluescript MSP3-12	Size of co-migrating plasmid and genomic fragments
Nco I	12/1,188	1,176
Eae I	52/1,762	1,710
Bsp M	193/2,076	1,886

according to the manufacturer's recommendations (The Genius System Luminescent Detection Kit, Boehringer Mannheim, Indianapolis, IN). The membranes were exposed to Hyperfilm - MP (Amersham International, plc, Buckinghamshire, England) to visualize bound antibody. The molecular sizes of comigrating cloned and genomic fragments were determined by comparison to Lambda DNA/Hind III fragments (GIBCO BRL, Gaithersburg, MD) and 1 kbp DNA Ladder (GIBCO BRL, Gaithersburg, MD) molecular size standards.

Presence of Multiple MSP3 Gene Copies

A. marginale genomic DNA from either the FL, VA, or SI isolate was extracted as described above and aliquots of 1 μg of DNA were digested with *Hinc* II (New England Biolabs, Beverly, MA), *Sac* I (Boehringer Mannheim Corp., Indianapolis, IN), *Nde* I (Boehringer Mannheim Corp., Indianapolis, IN), or *Sph* I (Boehringer Mannheim Corp., Indianapolis, IN). These enzymes were chosen because they do not cut within the known sequence of MSP3-12 gene. Calf thymus DNA was digested identically as a control. Empty pBluescript and pBluescript MSP3-12 DNA (0.1 μg) were digested with *Eco* RI and used as negative and positive controls, respectively for probe hybridization. Digested fragments were separated by gel electrophoresis with 1% agarose gels containing 0.1 μg ethidium bromide and photographed. Southern blotting was performed under prehybridization and hybridization conditions

as previously described above and blots were probed with digoxigenin-labeled pBluescript MSP3-12. Bound probe was detected by ECL as described above. Lamda DNA/Hind III fragments (GIBCO BRL,Gaithersburg, MD) and 1 kbp DNA Ladder (GIBCO BRL,Gaithersburg, MD) were used for molecular size standards.

Distribution of MSP3 Copies in the *A. marginale* Chromosome

Restriction Enzyme Digestion in Agarose Plugs

The locations of multiple MSP3 copies in the chromosome were determined by Southern blotting of large *A. marginale* genomic fragments separated by CHEF. Infected erythrocytes containing intact genomic DNA from FL, SI, and VA strains of *A. marginale* were embedded in 0.7% agarose plugs and stored in 0.5 M EDTA (pH 9.5), 1% N-lauroylsarcosine, and 2 mg/ml Proteinase K at 4 °C as previously described above. Agarose plugs were digested with *Not* I (Boehringer Mannheim Corp. Indianapolis, IN) and *Sfi* I (New England BioLabs, Beverly, MA) as previously described (Alleman et al., 1993). Briefly, each plug was washed in 10 times their volume of T.E (10 mM tris, pH 7.5, 0.1 mM EDTA) over several hours with fresh T.E replaced each hour, then incubated in T.E plus 1 mM phenylmethylsulphonyl fluoride (PMSF) for 2 hours at 37°C. Agarose plugs were then equilibrated on ice for 1 hour in 10 volumes 1x restriction enzyme buffer plus 0.1 mg/ml BSA. A

solution of 100 units of restriction enzyme in 1x restriction enzyme buffer was added and DNA digestions were performed according to the manufacturer's instructions. Reactions were stopped by the addition of 0.25 total reaction volume of 0.5 M EDTA, pH 8.0, 0.1% N-lauroylsarcosine (w/v), 1 mg/ml proteinase K (w/v), incubating reaction mixture at 4°C for 1 hour, then 37°C for 15 min. Uncut *M. bovis* chromosomal DNA (Promega Corp., Madison, WI) was digested identically to serve as a control for restriction endonuclease activity.

CHEF Gel Electrophoresis

This was done on the CHEF DRII system (Bio-Rad Laboratories, Richmond, CA). Plugs of digested DNA were electrophoresed in 1% agarose gels in 0.5x TBE buffer at 14°C. Electrophoretic conditions were set at 180 V, 10 sec. switch rate, with a 16 hour run time. Delta 39 Lambda Ladders (Promega Corp., Madison, WI) and Lambda DNA/Hind III Fragments (GIBCO BRL, Gaithersburg, MD) were used as size standards. The gels were stained for 30 min. in 0.5 μg/ml ethidium bromide and photographed. Southern blotting and hybridization of the separated bands were performed as previously described except that the gel was depurinated in 0.25 M HCl for 15 min. prior to washing and transfer to nylon membranes. Prehybridization and hybridization conditions were performed identically to those described above. The membranes were probed with digoxigenin-labeled pBluescript MSP3-12, and bound

probe was detected by ECL as before. The membranes were exposed to Hyperfilm - MP (Amersham International, plc, Buckinghamshire, England) to visualize bound antibody.

CHAPTER 4
RESULTS

Specificity Experiments

SDS-PAGE separated *A. marginale* initial body proteins were transferred to nitrocellulose, and probed with antisera from animals infected with related rickettsial agents or protozoal hemoparasites. This was done to determine if animals infected with these organisms contain antibodies which cross react with the 86 kDa protein (MSP3) of *A. marginale*. As a negative control, normal sera from various species were reacted with *A. marginale* proteins. Where available, protein preparations from related organisms were used in a homologous reaction with respective antisera to serve as a positive control.

Sera from a sheep infected with *A. ovis* (Fig. 3), a horse infected with *E. risticii* (Fig. 4), and a dog infected with *E. ewingii* (Fig. 4) showed strong reactivity with MSP3 of *A. marginale*. These sera as well as sera from animals infected with *E. equi* and *C. ruminantium* showed reactivity against other *A. marginale* antigens as well. Sera from animals infected with *A. ovis, C. ruminantium, E. ewingii,* and *E. equi*, showed reactivity against a 36 kDa antigen, possibly MSP2 (Figs. 3, 4, & 5). Sera from animals infected with *C.*

Fig. 3 Specificity experiments using immunoblots. *A. marginale* (AM) or *A. centrale* (AC) initial body preparations reacted with normal sera from non-infected sheep (NSS), non-infected cattle (NBS), or antisera from animals infected with *A. ovis* (AO), *A. centrale* (AC), or *A. marginale* (AM). Labeling above each lane indicates the serum used (top), the initial body preparation used (center), and the dilution of the serum (bottom). Molecular size standards in kilodaltons are illustrated on the left.

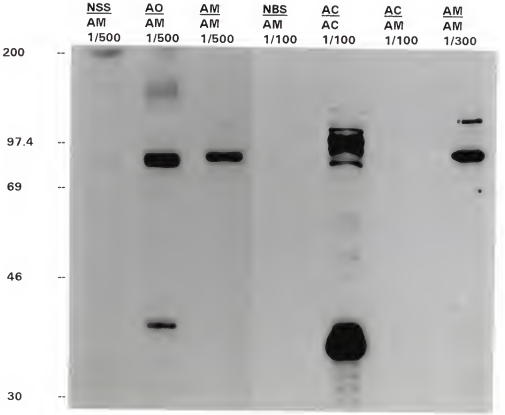

NSS
AM
1/500
AO
AM
1/500
AM
AM
1/500
NBS
AM
1/100
AC
AC
1/100
AC
AM
1/100
AM
AM
1/300
200
97.4
69
46
30

Fig. 4 Specificity experiments using immunoblots. *A. marginale* (AM) initial body preparations reacted with normal serum from a non-infected horse (NHS) or anti-sera from animals infected with *A. marginale* (AM), *E. risticii* (ER), *E. equi* (EE), or *E. ewingii* (EC). Labeling above each lane indicates the serum used (top), the initial body preparation used (center), and the dilution of the serum (bottom). Molecular size standards in kilodaltons are illustrated on the left.

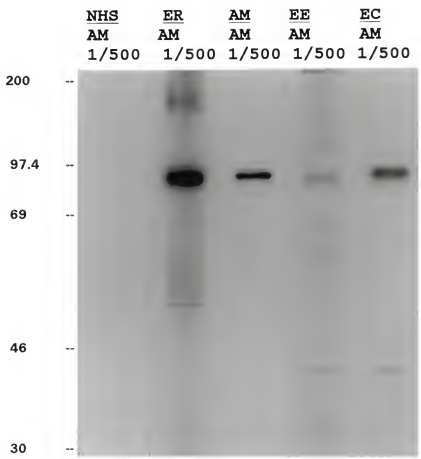
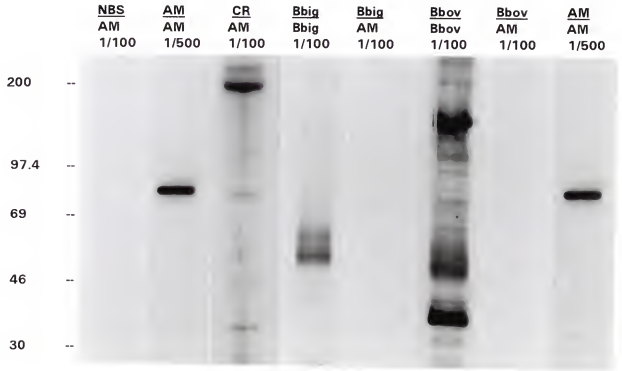

NHS
AM
1/500
ER
AM
1/500
AM
AM
1/500
EE
AM
1/500
EC
AM
1/500
200
97.4
69
46
30

Fig. 5 Specificity experiments using immunoblots.
A. marginale (AM) initial body preparations, or *B. bovis* (Bbov) or *B. bigemina* (Bbig) antigen preparations reacted with normal serum from a non-infected cow (NBS), or antisera from animals infected with *A. marginale* (AM), *C. ruminantium* (CR), *B. bigemina* (Bbig), or *B. bovis* (Bbov). Labeling above each lane indicates the serum used (top), the initial body preparation used (center), and the dilution of the serum (bottom). Molecular size standards in kilodaltons are illustrated on the left.


NBS
AM
1/100
AM
AM
1/500
CR
AM
1/100
Bbig
Bbig
1/100
Bbig
AM
1/100
Bbov
Bbov
1/100
Bbov
AM
1/100
AM
AM
1/500
200
97.4
69
46
30

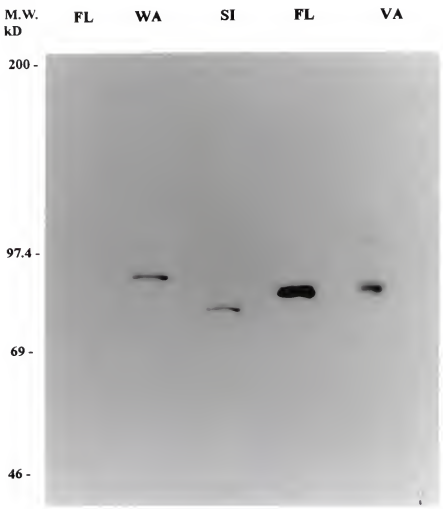
ruminantium (Fig. 5) and *E. equi* (Fig. 4) reacted with an antigen of slightly smaller molecular weight than MSP3. Sera from animals infected with *A. centrale* (Fig. 3) or either *Babesia sp.* (Fig. 5) failed to react with any *A. marginale* antigens although strong reactivity was demonstrated in reactions involving homologous preparations.

Conservation of MSP3 Between Different Geographic Isolates of *A. marginale*

The conservation of MSP3 between different geographic isolates of *A. marginale* was evaluated by SDS-PAGE and immunoblots using initial body preparations from FL, VA, SI, and WA isolates of *A. marginale*. Preparations were separated on 7.5% to 17.5% (w/v) gradient polyacrylamide gels as described above. They were then transferred to nitrocellulose, and reacted with varying dilutions of antiserum from an animal experimentally infected with a FL isolate and a MAb to MSP3, AMG75C2 (McGuire et al., 1991), for definitive identification of the MSP3 antigen. An optimal dilution of this antiserum was established for demonstration of the immunodominant MSP3 protein (Data not shown). Pre-infection bovine sera and a MAb to a trypanosome surface protein were used as negative controls and showed no reactivity to MSP3.

Initial body preparations from these same isolates were then probed with antiserum at a single dilution previously established (Fig. 6). The side by side location of the

Fig. 6 Size polymorphism of MSP3.
Immunoblots of initial body preparations from a Florida (FL), a Washington (WA), a South Idaho (SI), and a Virginia (VA) isolate of *A. marginale* (AM), reacted with normal serum from a non-infected cow, Lane 1 (Pre), or anti-sera from a cow infected with a FL isolate of AM, Lanes 2-5. Serum was diluted to 1/300. Molecular size standards in kilodaltons are illustrated on the left.



Pre Post

Anti-Fl A.M. sera (1/300)

various isolates clearly demonstrates variation in size of the MSP3 proteins. The 86 kDa MSP3 antigen is seen in the FL isolate (Fig. 6). An antigen of similar size is seen when this same antiserum is reacted with proteins from a VA isolate (Fig. 6). However, in reactions with the SI isolate, an antigen of slightly smaller molecular mass is seen, and the WA isolate has an antigen with a molecular mass greater than 86 kDa (Fig. 6).

Immune Response to MSP3

Realizing MSP3 is not conserved between different geographic isolates, we then investigated the possibility that animals infected with different isolates may contain immune sera that varies in reactivity to MSP3 from a single isolate. This is an important consideration in attempting to develop a diagnostic test antigen derived from a single isolate. Initial body preparations from a FL isolate were separated by 2-D gel electrophoresis as described above in order to determine if co-migration of antigens of similar molecular size occurs. Electrophoretically separated proteins were transferred to nitrocellulose and probed with antisera from cattle infected with a FL, VA, SI, or WA isolate of *A. marginale* as well as an anti-MSP3 MAb (AMG75C2), rabbit-anti-MSP3 polyclonal serum, and an anti-MSP2 MAb (ANF19E2). Pre-infection bovine sera, normal rabbit sera, and a MAb to a

trypanosome surface protein were used as negative controls and showed no reactivity to MSP3 (Data not shown).

In a homologous reaction with anti-FL serum, 2 major areas of reactivity were seen with a molecular mass of 86 kDa, one with an apparent isoelectric point (pI) of 6.5, and the other with a pI of approximately 6.2 (Fig. 7). There was slight reactivity with an antigen at a pI of approximately 5.6. When the initial body preparation from the FL isolate was reacted with the anti-MSP3 MAb, major reactivity was seen in the 5.6 area of the pH gradient, but no reactivity was noted with antigens at a pI of 6.5 or 6.2 (Fig. 7).

Serum from an animal infected with a VA isolate showed similar reactivity as the MAb. However, serum from an animal infected with a WA isolate reacted with 2 antigens in an entirely different area of the pH gradient, having pIs of approximately 5.1 and 5.3 (Fig. 7). When these same initial body preparations were reacted with antiserum from an animal infected with a SI isolate reactivity was noted in all 3 areas of the pH gradient, with approximate pIs of 6.5 to 6.2, 5.6 and 5.3 to 5.1 (Fig. 7). When a rabbit-anti-MSP3 polyclonal sera was used, reactivity was noted in areas of the pH gradient having pIs of 6.5 to 6.2 and 5.6 (Fig. 8).

Although conditions seen here were not optimized to separate the 36 kDa proteins of *A. marginale* (MSP2), multiple spots were visualized in that apparent molecular size (Fig. 7). Some variation in reactivity of the different antisera to

Fig. 7 2-D gel electrophoresis of *A. marginale* proteins. Immunoblots using initial body preparation of a FL isolate of *A. marginale* separated by 2-D gel electrophoresis. Letters centered above each immunoblot indicate the antibody used in the reaction, FL = antiserum from a cow infected with a Florida isolate of AM, VA = antiserum from a cow infected with a Virginia isolate of AM, WA = antiserum from a cow infected with a Washington isolate of AM, SI = antiserum from a cow infected with a South Idaho isolate of AM, and MAb AMG75C2 = anti-MSP3 MAb. Antisera are all used at a dilution of 1/300. Concentration of MAb = 5μg/ml. Numbers above each immunoblot indicate the pH taken at 1 cm distances along the length of the tube gel. Arrows indicate the isoelectric point of each 86 kilodalton band. On the far right side of each gel, initial body preparation from a FL isolate of *A. marginale* was electrophoresed in a single dimension and immunoblotted along with 2-D focused initial bodies to indicate the 86 kDa, MSP3. Molecular size standards in kilodaltons are illustrated on the left of each immunoblot.

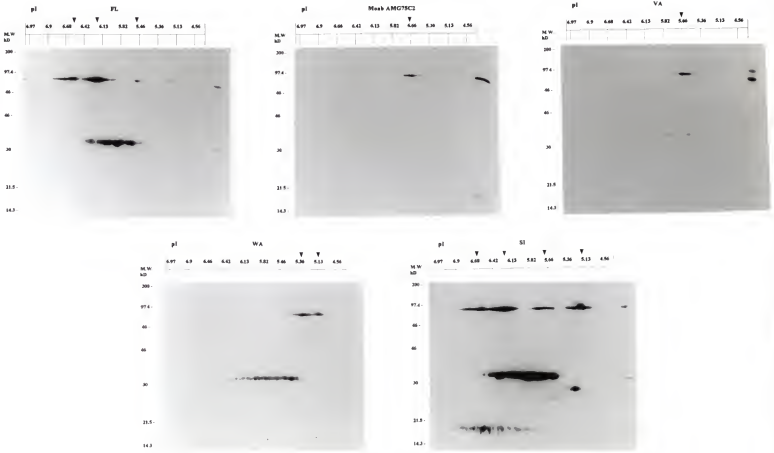
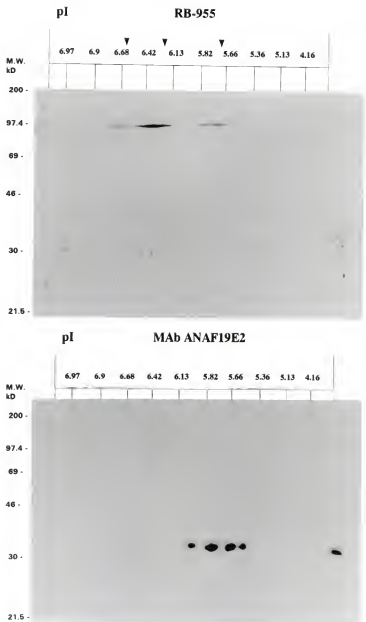

pI
FL
Mouk AMG75C2
VA
WA
SI
M.W kD
6.97 6.9 6.68 6.42 6.13 5.82 5.66 5.36 5.13 4.56
200
97.4
66
46
30
21.5
14.3

Fig. 8 2-D gel electrophoresis of *A. marginale* proteins.
Immunoblots using initial body preparation of a FL isolate of *A. marginale* separated by 2-D gel electrophoresis. Letters centered above each immunoblot indicate the antibody used in the reaction, RB-955 = rabbit anti-MSP3 polyclonal sera and MAb ANAF19E2 = anti-MSP2 MAb. Rabbit anti-MSP3 was used at a dilution of 1/5,000. Concentration of MAb = 5μg/ml. Numbers above each immunoblot indicate the pH taken at 1 cm distances along the length of the tube gel. Arrows indicate the isoelectric point of each 86 kilodalton band. On the far right side of each gel, initial body preparation from a FL isolate of *A. marginale* was electrophoresed in a single dimension and immunoblotted along with 2-D focused initial bodies to indicate the 86 kDa, MSP3 and 36 kDa MSP2. Molecular size standards in kilodaltons are illustrated on the left of each immunoblot.


pI
RB-955
6.97
6.9
6.68
6.42
6.13
5.82
5.66
5.36
5.13
4.16
M.W.
kD
200 -
97.4 -
69 -
46 -
30 -
21.5 -
pI
MAb ANAF19E2
6.97
6.9
6.68
6.42
6.13
5.82
5.66
5.36
5.13
4.16
M.W.
kD
200 -
97.4 -
69 -
46 -
30 -
21.5 -

MSP2 was seen. For example, in a homologous reaction there was reactivity with antigens with pIs from 6.42 to 5.66. However, when reacted with sera from animals infected with WA or SI isolates, reactivity with antigens with pIs of 6.42 to 5.36 was observed. These spots were confirmed to be the MSP2 antigen by use of an anti-MSP2 MAb (ANF19E2), the reactivity of which has been previously described (McGuire et al., 1984; Palmer et al., 1988; Palmer et al., 1994). Multiple spots with a molecular mass of 36 kDa were recognized by this MAb (Fig. 8).

MSP3 Nucleotide Sequence and Translation

Drs. Travis McGuire, Terry McElwain, and Guy Palmer (Washington State University, Pullman) identified and sequenced 3 clones of the MSP3 gene by screening a genomic library of a FL strain of *A. marginale* DNA with MAbs to MSP3. These clones were designated MSP3-11, MSP3-12, and MSP3-19. The 3 clones in pBluescript vectors, along with their nucleotide and deduced amino acid sequences, were supplied to us courtesy of the above individuals. The entire nucleotide sequence of each clone is illustrated in Figs. 9, 10, & 11. A complete gene sequence is lacking in each clone. However, MSP3-12 contains the N-terminus of the gene and 633 bp upstream to the open reading frame. This area upstream to the 5' end of the open reading frame is likely to contain

Fig. 9 Gene sequence of MSP3-11.
The nucleotide sequence of pBluescript MSP3-11 is illustrated. Numbers on the left indicate the number of the first nucleotide in each row. Termination codon is underlined.

Msp311.Conentry:1 Length:3263 May 29, 1991 11:34 Check:1828 ..

```
   1 GGTGGGGAGATGGTAGGAGTTGATGAAGGACTAGTTATACAAGAACTGAGCAGACCAGAAGAATTAGAAAAGCTACAACATGAACTAGCAAAGCAAGTAA
 101 GTAAATTAGCTGAACTTGGAGAACTTAAGTGGTTAGAGCAACTTGAGACACTGGAGACTGAGGAGTTGGAGAAGGTGGCGAAAAGAGCCACACAGAAGCT
 201 CAGTATATTGGAGGAGCAGCCAGGATACACCTCACTAGAGGAGCTGGAGAAGAAGCTGAAGGAGATAGAGGAAATAAGGAAACTGAAGGGACCGGAGGCA
 301 ATCAGAAAGCTTAGAGACTTGGAGAGGCTGAAGCTGAGAAGTTGGAGGAGGTGAAGAAGAAGGTAAAGGGTTCAGAGCTTGCAGACATTTGGACAAAA
 401 CGTGGTTGCTACGCAGGATTGGAAGAGTAGGGCAGGAACTAGCAGCGATAAGGGAGCTGAAGGAATTGGGGCTAGAGGAACGGCTCAGGGTACTAGCTGA
 501 GATTAAGGAAGTTAGAGCATTGGCAGAGAAGCGGAAGGCTGGGGGACTAGAGATTCAGGAGGGGTTACAGCTGACTGAGAAGATTAGAGCATTGGGTGGA
 601 CAGCTGGATCTGCTGGAGGCACGAATATTATTGGGGCTAGAAGCTGAGAAGATGAAGGAGGTGGAAGCGGCAAAGGAGGAAGTGGAGATTTTCCTGGGAT
 701 TGCTCCACAACAATGACACACAGACCAAGAAGATAGAGGAGGTGAAGACTCTGGTAATGCGGGTGGGTGACAGTCAGGGACTAAGGGGTCCGTTGGTCAA
 801 GAAGTTAGAGCAGCTGGCTAAGAAGTTGGAGCCAAAGGTGGGTGGCAATACAGGGTTTCAGGGTCAGGTAGGGCTAGTGAAGGATCTTAAGAAAAAGCTA
 901 GAAGAACTAGCAGCGATAAGGAAGCATTGGAGCAACTGAAGGTGGAACCAGCGCTGCTAGAAGGGATAAAGAGGGAGGTGCTAACACAGCAGAGTACTC
1001 AAGTTACACGTAGGGAGAATAATCCACAAAGCAAGGCAGAAGGTAAACTAGAAGAGATAAGAGCGCTTAGAAAGCTAGGACAGTTGGATGAGCTAATACC
1101 AGAAGAGAGATTGAGAGAATTGTCCAAGGTTAAGGAAACACTGAAGCGGTTGAAGGGTAAGGAGCTAGTAGACCTAGAGAGGAAGCTAGAAACTATGGGG
1201 GAACTAAAGCAGGAGATAGAGAAAATCAAGGGCAGGAAGACTTGAAGCAGCTAGAGGCTAGGAAGTTGGAGGAGGTGAAGAAGGCAAAAGGGAAGTGT
1301 TTATTCTGAGGACAAAGGAAAGTCTGGAGAAAGAGGGGAAGCTGGAGGAGTTAAGGGGCTATAAACTCAAGAAGGCGATAAAGAAGCTGGAAACCTTGAT
1401 AGAGAAGATACGAGGGGTCAGCACGCTGAAGGAACAGTGGGAGCCAAAAGTTGAGAAGCTCAAGAGCAAACTAGAAACACTAGCTGCCATAAGGGAGCTG
1501 AAGAAATTGGGGTTTAAAGATTGGTTGAGACTCAGGACACTAGAGGAGCTTACAGAGATAGCTGAGCAGCGTGGAGTTGCAACAGTGATGAAGGCAGCAT
1601 TAGCTAGTGCGATGGAGATAGCTAAGAACAGGGGTTGGACTGATTATTTAAACAGTCTAGATGTAAGTGAGAGACTAATGCTGCAAGGGAATTAATTGC
1701 TGCTGAGAAGATTAGAAAATGGGCTAGGGATATTAATAACTTGGATGCTGATGAACGGGGAATGGTCGCTGGGGCCCTAACCCCTTCTACAACAGTGTGA
1801 CTCACCACTCTCCCCCGAAACATCACTCACACTTCCAACTCTACTGGGCACCACCAAGGCAGCCAGCCTCTCTTGGCACCTCATCTCGTACCCTCTTGG
1901 CCTACCAAATCTCGACCCTGCCACCACAGCCCTCTGCACCGGTGTCACCTGCATAACCCGTCTCCCAGTCTCTTGTACCTCAACACCTGCATCTCATCTT
2001 CCTTACCCAATGTGGCATTCCACAATCTTTGTCCTCTCTACTTACCCCCTACTTCTTACCGGCACCACTCACTGCTTTCCCAGGTCAACTAGTCACCCAA
2101 CCTCTAGCACCTTCAGCATCTCAGCCCAACTCACCACAGCTCTCTGCACCTTCAACAGCTCTAGCAAAAGCCCCGGCAACAACTGCCTTATCCTCCTTAC
2201 TCAGGTTATTAATGTTATTGCCCATCCCCTGCAAGTTGATGGTGTCTTTGTTCCCCTCAACAGAAAGCAGTGTGTCTGTACCCTCAGTGAACACCTCACT
2301 AATCTTGGTGGTGGCAGTGCTATCGGTGGTACCACACTTCTTAGTGCCATTACTGCCTTGCCCTTGCCACACACATTCTGGCTCACCTTCTCACCATTA
2401 GTAGTACCCTCTACTGCTTACCCCACTTCTTGGCTTCACCCTTAGTCATCTTACCTAAAGCAGTGGCAAGACGGTCACTGACCTCTTGCTGTATCAT
2501 ATGCTAACTCCTTTCCTAATAAGAATACTGAAGCTGTATCCTCATTAGACTTCTTACCTCCCTTAATGACAAACCTTTCATACCCTACTTCTACTTCAAC
2601 CCTTGCTCCTCCAATACTATACCCAATGCTCCCCTCTAGGCTGTGAGCATGCTATGGTAGCCCTGATATGGTTATTCATCACAGCAGGTTCTACTAATG
2701 CCGATACTATCAGCAGTACTAACACTGCTGACGATACAGCTACCAAGGTTATTTGTGACGTCATTGTCTTCGTGCAGAAGCTGGGATTACCCATCATGAC
2801 AGGGGTGGTACTAGGTTCTGGCGTCATGGCTTGGCACACTTGCATGGCCTGCCATGGTAATGCTGGTTGTGTGTACAGCCATAGGTAAGCTTATGGCCAC
2901 TGTATTGAGGGTAATACCAGTAGTGCAAGGGTGGACGTAGAGGTTTTGGAGAGTGGTGATGTTGCTAAAACTTAGATTCTTACTCATTGCTGTAGTAT
3001 TAGCATTTGGTATCTTACATGGTGTACCTGCTGGGGCTAGTAAGTCCAGCGAGGGTGCTCAGACTGCTGATGATACAGCGACTGTTGTTATATGTAACGT
3101 TATACGGTTTGTGCAGAAGCTGGGACTACCACATCATGACTGGGGTGATACTAGGTCTACGATTATGGCTATCTTTGGTAAGCTTGCATGGCCTGCTATT
3201 GTAATGCTGGTTGTATTTACAGCTATATTCTTTGGTGCTGGTAAGCTTATGGCTAAATTCCCG
```

Fig. 10 Gene sequence of MSP3-12.
The nucleotide sequence of pBluescript MSP3-12 is illustrated. Numbers on the left indicate the number of the first nucleotide in each row. Start codon is underlined.

Msp312.Conentry:1 Length:2337 August 21, 1991 15:43 Type:N Check:1077

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   1 GGGGGCCTGCCATGGTAATGCTGGTTGTATGTACAGCCATAGGTAAGCTTATGGCCACTGTATTGAGGGTAATACCAGTAGTGCAAGGGTGGACGTAGA
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 201 AGTCCAGCGAGGGTGCTCAGACTGCTGATGATACAGCGACTATTGTTATATGTAACGTTATACGGTTTGTGCAGAAGCTCGGACTACCCATCATGACTGG
 301 AGTGATACTAGGTTCTAGCATTATGGCTATCTTTGGTAAGCTTGCATGGCCTGCTATTGTGATGTTGGTTGTATTTACAGCTATATTCTTTGGTGCTGGT
 401 AAGCTTATGGCTAAGTTTGCTGCTGGGTTGAGTGGTGAGGGTGTGAAGGATGCCGGTAGCTTTGACTGTTCCAGTTATAAAGGCACTGCTAGGCAGTGAT
 501 GGTGCAGAGAGCTGTGGTGGTCGTGCCAAGTTAGACTGGGATGCCAGGGGTTTATAGATAGTGGCTTTGTTATGTCGGTGGTACCAAGAACTTCAAGTGT
 601 GTTGGTGCCCAGTAGAGTTGCAAGTGTGAGTGATGTTTCGAGGGAGAGTGGTGATGATGCCAAAACTTAGATTCTTACTGATTGCTGGGGTATCAGCATT
 701 GGGTATCCTCCATAGTATGTCTGCGGGGGCTGCCGATAGAAGTAAGTCAGCACACAGAGGTTGGCTAGTGGACCGGCTTTGGGGAAAGGCAACGGGAGC
 801 TTCTACATAGCCCTAGACTATAACCCAACTTTCAACGGTATCAAGGACCTGAAAATCATCGGCGAAACCGATGAGGATGAAATGGATGTCTCACCGGTG
 901 CCAGGGGCCTGTTCCCGATGAACGCTCTTGCTAGCAACGTCACCGATTTTAACTCATCACCACTTCGACTGGAGTACCCCACTGCCTGGGCTAGAATTTGG
1001 GAACAGTACCCTGGCTCTTGGAGGAGCATTGGGTACAGAATTGGAGGAGCCAGGGTTGAAGTAGGGATAGGACATGAGAGGTTTGTTATTAAGGGGGGA
1101 GATGATGCAGCATTCCTACTAGGTAGGGAACTAGCATTGGATACAGCGAGGGTCAGTTACTATCCAGTGCATTGGGTAGGATGTCCATGGGTGATGTAC
1201 ACAGATTAAAGAAGGAAGTAGTTGATAGTATAGGAAGAGGAACAGCTAGTCCTGTAAGGGCAATGTTTAGTAGAGAGATCTCAGATGGGAATACATTACT
1301 TGCTGGGGAGATGGTAGGAGTTGATGAAGGACTAGTTATACAAGAACTGAGCAGACCAGAAGAATTAGAAAAGCTACAACATGAACTAGCAAAGCAAGTA
1401 AGTAAATTAGCTGAACTTGGAGAACTTAAGTGGTTAGAAGAACTTGAAAAGCTGGAGACTGAGGAGCTGGAGCAAGGACTCGAAGGAGCGCTAAAAGCTT
1501 TGGGCGTGGAAGCATCAGTGCAGGAGCTGGTACAGAGATTCAAGAAGGAAATTGCTGATGGTAAGACACCGGAAGAGATAAAGCTGGAGTGGATCAAGGA
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1701 GAAATACGGGAAGTTAGGCAGCTCAAGGAACTAGCGGATAAAGGAGGAGCGTTAGGAATCATGGCCGAGAAGCTCAAGAAGCAGGAAAGTCTCAAGGGGC
1801 TAGGAGGAACAGTAGAAGAATTAGCAGCGATAAGGGAACTGAAGGAATTGGGGCTAGAAGAGCAGCTGAGGATGCTGGCTGAGATTAAGGAAGTTAAGAG
1901 ATTGGCAGAGAAGCAGAAATCACAAGGATTAGAGGCTCATGAGGGATTACAACTGACTGAGAAGATTAGAGCATTGGGAGGACAGCTGGATCTGCTGGAA
2001 GAGAGATTGAGTGAAAAGTTTAAGAGACTACAGCAGAATGAGCAAGGTATACGTAAGGATCTGAATCCACAAGCTATAACAGCAGTGGCAGGTAAACTAG
2101 ATGAGATTTGGGTACTGGGGACGCTAGGACAGTTAGATGAGCTAGTGCCTGGGAAGAGAGCTCAGACACTGGGGGAAATGAAGGCAACGCTGAAGGAGCT
2201 CGAGAAAATACGGAAGTTAGTAGACCTAGAGAAGAAGCTAGAAACTATGGGAGAACTAAAGAAGGAGATAGAGAAAATCAACGAGGAGGCAGAGCTTGTG
2301 AGGTTTACACGCAAAGAAGTCTCGGAGATATCACCCG
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Fig. 11 Gene sequence of MSP3-19.
The nucleotide sequence of pBluescript MSP3-19 is illustrated. Numbers on the left indicate the number of the first nucleotide in each row. The termination codon is underlined.

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Msp319.Conentry:1  Length:4185  October21, 1991  15:01  Type:N  Check:2553
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 201 AAAGCAAGTAAGTAAATTAGCTGAACTTGGAGAACTTAAGTGGTTAGAAGAACTTGAAAAGCTTGGAGACTGAGGAGTTGGGGGAGCTGTTGAGACTGAAA
 301 GCGAGAAAAGCCTCACAGGAACTTAGGACGTTGGCGGAAAGTAAGGACAGCACCTAAATGCTGATGAGATAGAGGGGGAACTAAAGAGCGCGCTGGGAT
 401 TGGAAAGCTGGAAAAGTTGCCAGAGCTGGAGTTGGTCAAGCAGCGGCTGAGTCGATGAAGGAGCTGGAGAAGAAGATAGAGGAGGCGCAGTTGACAGC
 501 AGAAGAGCTCGGGGAAATGAGGGAGAAGCTTAAGGAGTTGCCAGGAAGGGGGGAGGATCCTGCGGGGCTGAAGAAAGCGATAGAGGAGGCAAATGCTGAC
 601 GAACTCAAGAAGGGGCTGAAGGAGGTGCTGAAGACGCTGGAGGGAAAGAAGTCAGAGCTGGTGAGGGAAGTAGGAAGGTTGAAGAAGGGAGCCTTGGAGG
 701 AGATGAAGGGAGGAAAGGGGTTGGTAGAGGAGGTAAAGGGAGAGCTAGGAAAGCTAGAGGCTAAGAAGATAGAGGAGGTGAAGGCGGCAAGGGGGAAGT
 801 GTCGTTTTTCCCAGGAATAAGAGAACTCACCAAAACTGGAAAGGAGTCGGAGTTGCTGGAAAGGCTTGAGGAGGGCTGCAGGCGGCTAAGAAGATAAAG
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1001 TGCTCAAGGAGATAAGACTGGAGCTGGAAGAGCAGCTGGGATGCTAGCTGAGATTAAGGAAGTTAAGGCACTGGCAGAGAAGCGGCAGGCTGGAGGACT
1101 GGAACTTCAGGAGGTATTACAGCTTACAACTAGAACTGTGCCATTGGAGAGGAAGCTGGTAGAAAAGGTAAGTAAGCTAGATAGGAAGAGCCTGGAAAGT
1201 CAGAAGAAGGTACTGGAGGAGCATAAGAAGGAGCTGGAGCAGGAAAAGCTTAAGCTAACTAAGGAGCTGGCTGAGAAGCGAAAAGCCAAAGGGCTCTTG
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1701 AGTGTGACTCACCACTCTTCCTCGCAACATCGCTCACACTCCTAACCGGCACTGTACTGAGGGTAATACCAGTTAGTGCAAGGGTGGACGTAGAGGTTT
1801 TGGGAGAGTGGTGATGTTGTCAAAACTTAGATTCTTACTCATTCGTGAGATTAACGATTGGGTATCTTACATGGTGTACCTGCAGGGGCTGCTGCTCAG
1901 GCGAGCCCTAGTACTACTGGTACTGAAGCTGATGATACAGCGACTGTTGTTATATGCAATGTTATACGGTTTGTGCAGAAGCTGGGACTACCCATCATGA
2001 CTGGGGTGATACTAGGTTCTAGCATTATGGCTATCTTTGGTAAGCTTGCATGGCCTGCTATTGTAATGCTGGTTGTATTTACAGCTATATTCTTTGGTGC
2101 TGGTAAGCTTATAGGCTAAATTTGCTGCTGGGTTGAGTGGTGATGGCATAGGTGATGCCAGTAAGTTTGACTGTTCACAGCATACAGCGGTCAACACCAGC
2201 CAAAGTAACCGATAGAAGATTAGGTGCTGCTGGGCAGTGATGGTGCAGAGAGCTGTGTTGCATTGGTTGGGAGATGAGGCAGCGGGAGTTCGGAGATAG
2301 GGACTAGGTCATGGACGATGCAGGTGTGGGCAGAGAGCTGTGTTGGCCTTGGTAGTGAGTTGGGCTGAGGTGCCAAGGGCAGGAGGAGGTACCAGAAGT
2401 TGGGAGATGCTGGTTACCGTTGAAGTGAACCGGTGCAGAGAGCTGTGGCCTTGGGGCCCAGTAGAGTTGGGCTGAGGGGCCAGGGGTAGTGAAGGACAG
2501 ATTTGACTGTTCCAGTTATAAGGAAGTCAAGACCGGCACCAAACCAAGCTAAAGGTTAGGAAATGAAGTGCCGCGTGATGGTGCAGAGAGCTGTGGTGGT
2601 AGTACAGGGGTGCTGGTGATAGTTCTGGTGCTGCAGGGGATCAGAGGACCTCACTACTGCTGCGGATTATCTCCTTTTGTGTTCCCCACAGGTTCACAT
2701 AATTAACCGTACATGGGTAAATGTGCTGGTAGTGCCGGGAACCGCAAGTGGGTAGATGGTGGAGGGGCACCATTGGGGTGGGTGTCCTAGAATCCGAACC
2801 TGATTCCTAGTTCTACCCCTATATTGCGTAGCCCGAAGGATGCTGAGACCTTCTCCTTGTTTTCCTAATGGGCTGGCGTCATCGACTCACGGTGGGC
2901 AGGGGTGTTGTCGTAGTTTGTGCCTAGTGTTTGCTGGCAGTGCCGCCAATGAATGCTGCCAGGCTGGGTGTGATGCTGTAACTCACACCAGCCTTGACC
3001 TGGCAGGTAAGCTGTGGCTGAAATCGTTGGGCGGTCAGTCCTACAAAGCTTGCACCCAACCCTGCGCAGGCGTAGGGAGACATTCTCCAGTCAACTAGTA
3101 ACCCCACCTGTGGGAAATCATAGCATCGACTCAGGATCGGTGAAACTACCCTGACCCCACTTATTTCCACAATCTCCATGCCCTCTGTGCTTGTGGCAAT
3201 TGCCGACCAATGACGCCTTTTGCGAGCCGTGAGAGGCCAACAATATCCGTGATAGCTTGATTCCGCTTGCTCTCTTGCGTGTTCTGTGCAGAGGTATCA
3301 GCGCTACCAGCACCGGTTGCAGCAGCACGTTCAGTTTTGGCCTCCTCCCCAGGTATGATGCATCAAACTTACTTATAACCTTGTCAGCAAGCGCTTTGC
3401 TTTCCCACGCTTCGTCATGGTCTCTAATATCTTCCAAACGCTTCTTGATGGATCTCAAAGCCGGCTTGGACAACTCCTTCTGCAGCGCGGCCCGAAACCT
3501 TCGACGTCACGCGTCGCACTGGCAGATATTCTTTTCACCAGGGCAAAAGTCGCACTCCCCGCCGCTCAGTAAGGCATAATTTTGCGATTTGAAATCATAGT
3601 GCTCATGTCCGAATTCTAACTCAAGCCTGGTGCCGCGTACCACGCAACCCACGCTTCCCCTGAGGCCCAACAGACTGCTGTCTCAAACCCCATGGCCGG
3701 ACTCTCCGTAGATTCCCCCGCCCAATCGTAGTTGGCGGAAGAAAGCTCAGCAGAGCCCAACTTCCCAATGTAGGGCAGTATCGCGACGGTTTCTTTTGCA
3801 GCATTAAGCCTAAACCCGCTTATCTTCCCTATGGTGGGCCCGTACCCAAACTCACGTAAAAATCTTCACGACCGTTCATCCCATGGGAACTCGCTGGGA
3901 CCAGATAACCAATCGTACTCAGCAATGCAGCAGCTATACATACTACGGGCTCATGACGCACCCAATGACCCGACAACCAACAGGAGGCTAAACGCCCC
4001 TCACTGCACGACAGTGCTATACAACTGTTAAAAAGGTCTTAAAATGAGTTGCATTTTTGAAGGGCTTGCAACTATGCCTAGCTAGAGAATAAGGATTCCG
4101 GGCTGGCGGGCCAAACGGCGCACACTCAGGTTGCCCTTCGTGTCATGCATCATCAGCGTGGCACTACCTACTTTTTCAGCACCG
```

regulatory sequences for gene expression. Clones MSP3-11 and MSP3-19 lack the N-terminus but each contains the C-terminus and 1,473 bp and 2480 bp respectively, downstream to the 3' end of the open reading frame. A schematic representation of each clone shows areas of homology between the 3 genes as well as areas which are homologous to the MSP2 gene of *A. marginale* (Fig. 12).

Homologous areas are distributed throughout the 3 MSP3 genes. Areas common to all 3 genes are at bases 1191 to 1472 and bases 1818 to 2008 of the MSP3-12 clone. An area at the 3' end of MSP3-12 between bases 2090 to 2280 is also found in MSP3-11, but is absent in MSP3-19. The last 200 bp at the C-terminal end of genes MSP3-11 and MSP3-19 are homologous. This area is unavailable for comparison in MSP3-12.

The MSP2 gene encodes a 36 kDa surface protein, and is known to be expressed by a polymorphic, multigene family (Palmer et al., 1994). Areas of homology between the N-terminus of the MSP2 gene and MSP3-12 are indicated in Fig. 12. The N- terminus is absent in the MSP3-11 and MSP3-19 clones, so comparisons cannot be made. The areas of amino acid sequence homology between MSP3-12 and MSP2 (amino acids 55 through 176) shows 65.6% similarity and 54.9% identity (Fig. 13). There is a homologous area of over 500 bp between MSP3-11 and MSP2 (Fig. 12). However, this area is outside of the open reading frame, in a different reading frame, and a

Fig. 12 Diagram of MSP3 clones with MSP2 homology.
A schematic representation of clones MSP3-11, MSP3-12, and MSP3-19. Homologous regions are indicated by like shaded areas. Regions homologous to the MSP2 gene are indicated by MSP2->. Arrows indicate the direction of the reading frame. Nucleotide numbers are indicated on the bottom. Top numbers of clones MSP3-11 and MSP3-19 indicate corresponding nucleotides in clone MSP3-12.

MSP3 CLONES

MSP3-12

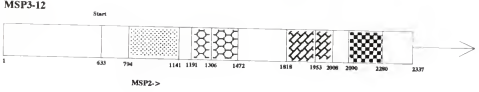


Fig. 13 Comparison of MSP3 and MSP2 protein sequences. BestFit alignment of amino acid sequences of MSP3 (top) and MSP2 (bottom). Identical amino acids are indicated by a vertical line. Conservative substitutions are indicated by an asterisk (*). The symbol ..... is used to denote a gap used to achieve optimal alignment between the sequences. Amino acid numbers are indicated at the beginning and end of each line.

BestFit Allignment of MSP3-12 (top)
and MSP2 (bottom)

```
55  GSFYIGLDYNPTFNGIKDLKIIGETDEDE 83
    ||||||||||.| |  ||| |.  |
50  GSFYIGLDYSPAFGSIKDFKV.QEAGGTT 77

84  MDVLTGARGLFPMNALASNVTDFNSYHFD 112
          ||.||     |.   ||  ..||
78  .......RGVFPYKRDAAGRVDFKVHNFD 99

113 WSTPLPGLEFGNSTL.ALGGSIGYRIGGA 140
    || | | . | .| | || |||||  ||||
100 WSAPEPKISFKDSMLTALEGSIGYSIGGA 128

141 RVEVGIGHERFVIKGGDDAA......FLL 163
    |||| .|.|||||||||  .        |||
129 RVEVEVGYERFVIKGGKKSNEDTASVFLL 157

164 GRELALDTARGQL 176
    |.|||  |||||.
158 GKELAYHTARGQV 170
```

different direction. A similar, but much smaller area is seen downstream to the open reading frame of MSP3-19 (Fig. 12).

MAb and Immune Sera Reactivity to Recombinant MSP3

Lysates from *E. coli* cells transformed with pBluescript containing each of the 3 MSP3 genes were separated by SDS-PAGE, transferred to nitrocellulose and reacted with anti-MSP3 MAb (AMG75C2) or immune sera from cattle infected with a VA or FL strain of *A. marginale*. This was done to verify that a cloned MSP3 gene represented a gene which produced one or more of the 86 kDa antigens seen on 2-D immunoblots. Initial bodies from a FL strain of *A. marginale* were used as a positive control for MAb (AMG75C2) and immune cattle sera. Lysate from *E. coli* transformed with empty pBluescript, anti-*Trypanosome brucei*-MAb (TYRP1E1), and pre-immune cattle sera were used as negative controls.

MAb AMG75C2 reacts only with recombinant protein expressed by clone MSP3-12 (Fig. 14). The recombinant MSP3 has a molecular size of approximately 65.0 kDa. This corresponds to the calculated size of the protein expressed by the open reading frame of MSP3-12 which is 1,707 bp (Fig. 10). No reactivity was observed between MAb AMG75C2 and recombinant MSP3-11, MSP3-19, or empty pBluescript. Isotype control MAb TRYP1E1 showed no reactivity as well.

Immune serum from a cow infected with a VA isolate of *A. marginale* reacted with recombinant MSP3-12 (Fig. 15).

Fig. 14 Immunoblots of expressed MSP3 clones.
The reactivity of expressed proteins from clones MSP3-11, MSP3-12, and MSP3-19 are compared using an anti-MSP3 monoclonal antibody (AMG75C2) and a negative isotype control (TRP1E1). pBlue is expressed proteins from empty pBluescript plasmid used as negative control. FL is *A. marginale* initial body preparation from a FL isolate as a positive control. Numbers on the left indicate molecular size markers in kilodaltons.

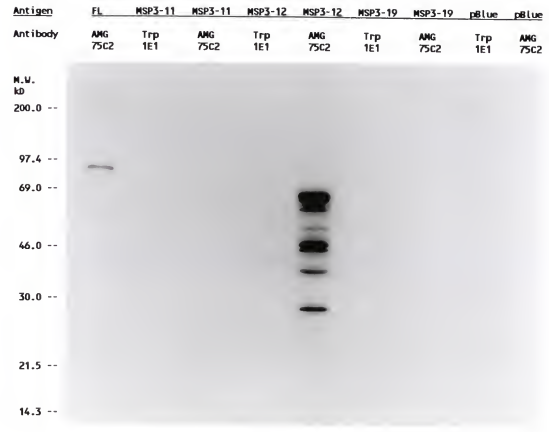
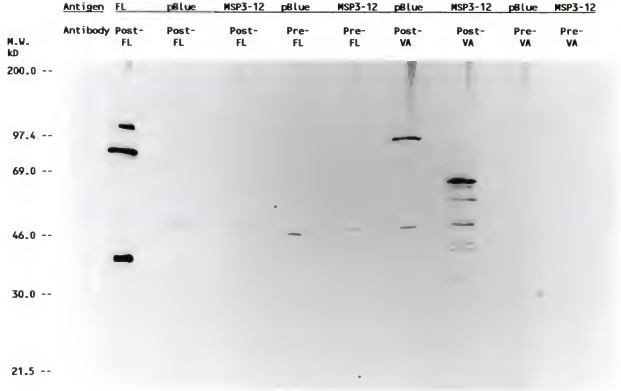

Antigen
FL
MSP3-11
MSP3-11
MSP3-12
MSP3-12
MSP3-19
MSP3-19
pBlue
pBlue
Antibody
AMG 75C2
Trp 1E1
AMG 75C2
Trp 1E1
AMG 75C2
Trp 1E1
AMG 75C2
Trp 1E1
AMG 75C2
M.W. kD
200.0
97.4
69.0
46.0
30.0
21.5
14.3

Fig. 15 Immunoblots of expressed MSP3-12 clones.
The reactivity of pre and post-infection sera from animals infected with a Florida isolate of *A. marginale* (Pre and Post-FL) or a Virginia isolate (Pre and Post-VA) are compared using expressed proteins from clones MSP3-12 and empty pBluescript plasmid (pBlue). Antigens are listed on top and anti-sera is listed on the bottom. In lane 1, initial body preparation from a FL isolate of *A. marginale* (FL) is reacted with immune sera from an animal infected with a FL isolate as a positive control. Numbers on the left indicate molecular size markers in kilodaltons.


Antigen FL pBlue MSP3-12 pBlue MSP3-12 pBlue MSP3-12 pBlue MSP3-12
Antibody Post-FL Post-FL Post-FL Pre-FL Pre-FL Post-VA Post-VA Pre-VA Pre-VA
M.W. kD
200.0
97.4
69.0
46.0
30.0
21.5

However, when this same recombinant protein was reacted with immune sera from an animal infected with a FL isolate, no reactivity above that which was seen with empty pBluescript was observed.

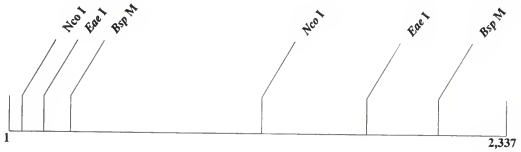
Representation of pBluescript MSP3-12 in the *A. marginale* Genome

To verify that the cloned pBluescript MSP3-12 faithfully represented the genomic copy, genomic DNA from a FL isolate of *A. marginale* and pBluescript MSP3-12 DNA were digested with restriction enzymes to yield predicted fragments of specified lengths. The enzymes used, the restriction sites in pBluescript MSP3-12, and the predicted size of the fragments are illustrated (Fig. 16). DNA fragments were Southern blotted to nylon filters and probed with whole digoxigenin-labeled MSP3-12. All enzymes used produced pBluescript MSP3-12 fragments of the predicted size which comigrated with a fragment in the genomic DNA (Fig. 17). Comigration of large fragments was seen in digests extending from the 5' terminal region to the 3' terminal region of the gene.

Presence of Multiple MSP3 Gene Copies

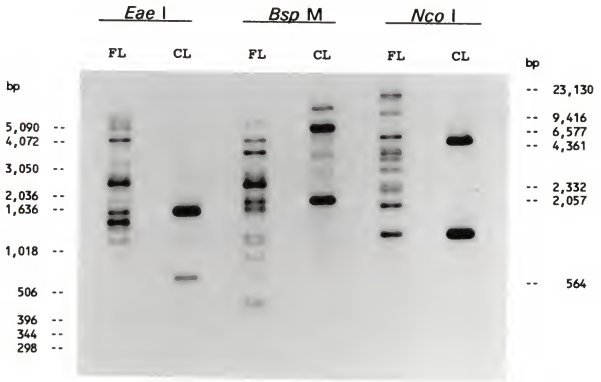
A. marginale genomic DNA from either FL, SI, or VA isolates was digested with restriction enzymes, Southern blotted to nylon membranes, and probed with whole, digoxigenin-labeled MSP3-12. Enzymes *Sph* I, *Nde* I, *Sac* I, and

Fig. 16 Representation of clone MSP3-12 in the *A. marginale* genome. Diagram of clone MSP3-12 indicating the restriction enzyme sites and the resulting fragments used to map MSP3-12 to the genome. Nucleotide cleavage site for each restriction enzyme is illustrated diagramatically (top) and by nucleaotide number(bottom). Size of resulting fragments in base pairs is also provided.



Restriction Enzyme	Cleavage site in pBluescript MSP3-12	Size of co-migrating plasmid and genomic fragments
Nco I	12/1,188	1,176
Eae I	52/1,762	1,710
Bsp M	193/2,076	1,886

Fig. 17 Genomic representation of MSP3-12.
Southern blot of genomic DNA from a Florida (FL) isolate of *A. marginale* or pBluescript MSP3-12 (CL), digested with restriction enzymes *Eae* I, *Bsp* M, or *Nco* I and probed with digoxigenin-labeled MSP3-12. Molecular size markers in base pairs (bp) are indicated to the right and left.


Eae I
Bsp M
Nco I
FL
CL
FL
CL
FL
CL
bp
bp
5,090
4,072
3,050
2,036
1,636
1,018
506
396
344
298
23,130
9,416
6,577
4,361
2,332
2,057
564

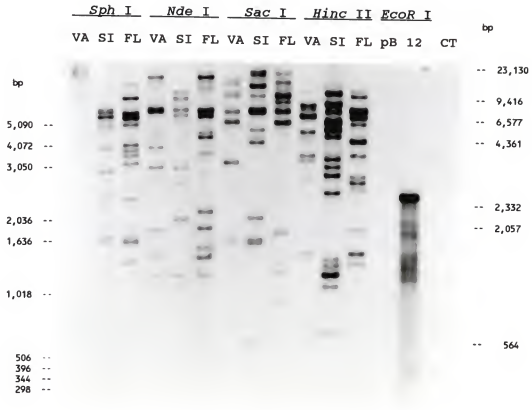
Hinc II were chosen because they do not cut within the sequence of the MSP3-12 gene. This should produce a single band if only one genomic copy of MSP3 is present. *Sac* I cuts once within the open reading frame of MSP3-19 (at nucleotide 498), potentially producing 2 observable bands for a single copy gene. None of the enzymes cut within the MSP3-11 gene.

Multiple bands were observed on Southern blot hybridizations, indicating multiple partially homologous MSP3 copies (Fig. 18). The exact number of copies cannot be determined since restriction site polymorphism may exist in other MSP3 copies, resulting in more than one band from a single copy. Similar intensities of many of the bands within a single isolate may indicate extensive homology exists between some of the copies. Restriction fragment length polymorphism (RFLP) is seen when comparing enzyme digests of each of the 3 isolates of *A. marginale*.

Distribution of MSP3 Copies in the *A. marginale* Chromosome

Intact, *A. marginale* genomic DNA from 3 isolates (FL, SI, & VA) was digested into large fragments with restriction enzymes *Sfi* I and *Not* I, and separated by CHEF electrophoresis. The gel was stained and photographed (Fig. 19). These fragments are nonoverlapping and have been previously shown to represent the entire 1,250 kbp *A. marginale* genome (Alleman et al., 1993). The *Sfi* I and *Not* I

Fig. 18 Presence of multiple copies of MSP3.
Genomic DNA from a Virginia (VA), South Idaho (SI), or Florida (FL) isolate of *A. marginale* was digested with the restriction enzymes indicated above the lanes and Southern blotted. *EcoR* I digested pBluescript MSP3-12 (pB 12) and undigested bovine calf thymus DNA (CT) were used as controls. The blots were hybridized with digoxigenin-labeled pBluescript MSP3-12. Molecular size markers in base pairs (bp) are indicated to the right and left.


Sph I
Nde I
Sac I
Hinc II
EcoR I
VA SI FL VA SI FL VA SI FL VA SI FL pB 12 CT
bp
5,090
4,072
3,050
2,036
1,636
1,018
506
396
344
298
bp
23,130
9,416
6,577
4,361
2,332
2,057
564

fragments produced here are identical to those previously reported (Alleman, et al., 1993). *Sfi* I digestion of the FL isolate produced 12 bands ranging in size from 170 to 14 kbp.

This same gel was then Southern blotted to nylon filters and probed with whole, digoxigenin-labeled MSP3-12 (Fig. 20). The probe hybridized to multiple fragments of *Not* I and *Sfi* I digests of all 3 isolates. Most of the gene copies in the FL isolate appear to be contained within 2 large *Sfi* I fragments with previously reported band sizes of 170 kbp and 137.5 kbp (Alleman et al., 1993). However, the results do indicate the MSP3 gene is widely distributed throughout the genome in all 3 isolates tested. Gene copies were present on 6 of the FL and VA *Sfi* I digested fragments and 5 of the fragments from the SI isolate (Fig. 20). *Not* I digestions yielded 8 bands in the FL isolate which hybridized to the MSP3-12 probe, 7 bands in the VA isolate, and 5 bands in the SI isolate (Fig. 20). RFLP is also observed between isolates on CHEF gels.

Fig. 19 *Not* I and *Sfi* I digestion of the *A. marginale* genome.
Genomic DNA from a Florida (FL), Virginia (VA), or South Idaho (SI) isolate of *A. marginale* was digested with the restriction enzymes indicated above the lanes, separated by clamped homogeneous electrical field gel electrophoresis, and stained with ethidium bromide. Lanes 1 and 2 contain lambda DNA-*Hind*III fragments and Promega Delta 39 markers, respectively, as size markers. Molecular size markers in kilobase pairs (kbp) are indicated to the left.

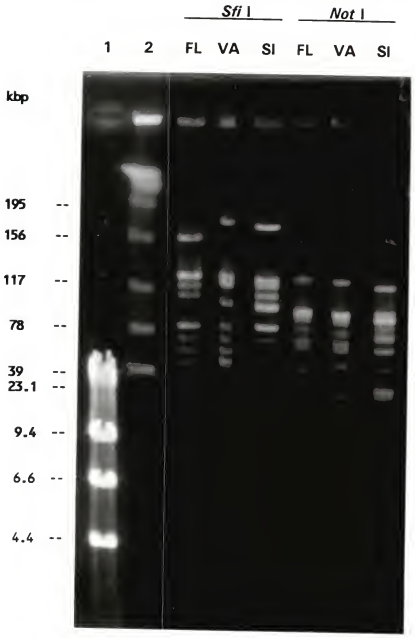
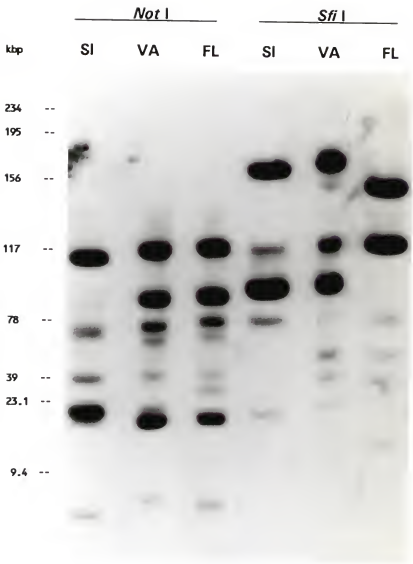

Sfi I
Not I
1
2
FL
VA
SI
FL
VA
SI
kbp
195
156
117
78
39
23.1
9.4
6.6
4.4

Fig. 20 Distribution of MSP3 in the *A. marginale* genome.
Genomic DNA from a South Idaho (SI), Virginia (VA), or Florida (FL) isolate of *A. marginale* was digested with the restriction enzymes indicated above the lanes, and separated by clamped homogeneous electrical field gel electrophoresis. The fragments were then Southern blotted and probed with digoxigenin-labeled pBluescript MSP3-12. Molecular size markers in kilobase pairs (kbp) are indicated to the left.


Not I
Sfi I
kbp
SI
VA
FL
SI
VA
FL
234
195
156
117
78
39
23.1
9.4

CHAPTER 5
DISCUSSION

Rational for Studying the MSP3 Antigen

The current serologic tests available for the diagnosis of *A. marginale* infection are based on antigens which are a crude mixture of *A. marginale* and erythrocyte proteins. This reduces both the sensitivity and specificity of various tests to unacceptable levels, particularly in detecting carrier cattle (Goff et al., 1990; Luther et al., 1980; Maas et al., 1986; Todorovic et al., 1977). Because of this, and because *A. marginale* cannot be successfully maintained in erythrocyte culture, efforts using molecular techniques have been attempted to identify an immunodominant protein with acceptable sensitivity and specificity to be used in recombinant form as purified test antigen. The ideal test antigen would be one which is not cross reactive with antigens from related organisms, and could detect acutely infected animals as well as carrier cattle infected with any of several different geographic strains of *A. marginale*. This antigen should preferably be conserved in all isolates of *A. marginale* and be from a single copy gene to limit genetic recombination and antigenic variation.

One antigen identified as immunodominant in the acutely infected animal as well as chronically infected carriers is the 86 kDa MSP3 protein from a FL isolate of A. marginale (McGuire et al., 1991; Palmer et al., 1986). The FL isolate of *A. marginale* was used for antigen isolation because it has been found by adsorption studies to contain antigens common to both morphologic types of *A. marginale*, the tailed and non-tailed forms (Goff and Winward, 1985; Kreier and Ristic, 1963). Experiments using affinity purified MSP3 showed excellent test sensitivity, however, attempts to produce a recombinant form of MSP3 have resulted in inconsistent responses to immune cattle sera. Possibilities for these inconsistencies include 1) an inability to produce an antigenically similar recombinant form of this protein in the chosen vector (*E. coli*), or 2) variants of MSP3 exist within or between different strains of *A. marginale*. Antigenic variability has been previously identified in another major immunodominant surface protein of *A. marginale*, MSP2 (McGuire et al, 1984; Palmer et al., 1994). In these experiments, not all organisms between strains, or within the same strain reacted with a given MAb to MSP2.

In addition, the specificity of MSP3 in detecting *A. marginale* infected cattle has not been totally established. Previous experiments have determined there is no cross reactivity between MSP3 when reacted with sera from animals infected with *B. bovis*, *B. bigemina*, or an unidentified

rickettsial agent isolated from an aborted calf fetus (McGuire et al., 1991). However, using 16s rRNA sequencing analysis, *A. marginale* has been shown to phylogenetically be more closely related to *C. ruminantium* and the *Ehrlichia sp.*, particularly *E. risticii, E. equi,* and *E. canis* (Dame et al., 1992; Van Vliet et al., 1992).

Initial experiments suggested FL MSP3 was conserved enough to be recognized by sera from animals infected with different isolates (Palmer et al., 1986). However, since this study used single dimension gel electrophoresis, reactivity of immune sera with different proteins of comigrating molecular size could not be ruled out.

The specificity of MSP3 in detecting *A. marginale* infected carriers, the conservation of MSP3 between various strains of *A. marginale*, the reactivity of sera from animals infected with different strains of *A. marginale* to MSP3 from a FL isolate, and the gene(s) which encode the MSP3 antigen needed to be investigated in order to determine if a recombinant form would make a suitable diagnostic test antigen.

The Specificity of MSP3 in Detecting *A. marginale* Infection

Antigenic similarities between *Anaplasma sp.*, particularly *A. marginale* and *A. centrale* have previously been identified (Palmer et al., 1988a; Shkap et al., 1990; Shkap et al., 1991). In our study, *A. centrale* antiserum reacted with

several *A. centrale* proteins, however, this same antiserum showed no reactivity against *A. marginale* antigens. In previous experiments, strong reactivity was seen with the 36 kDa protein (MSP2) of an Israel isolate of *A. marginale* when reacted with immune sera from a cow infected with *A. centrale* (Shkap et al., 1991). In addition, epitopes common to both species were identified using MAbs against the 36 kDa and 105 kDa surface proteins of *A. marginale* (Palmer et al., 1988a). Discrepancy between the results of this study and previously reported data may be explained by differences in geographic isolates of *A. marginale* used. Antigenic differences between different isolates of *A. marginale* have been well documented (McGuire et al., 1984). In our study, a FL isolate was used, whereas an Israel isolate of *A. marginale* was used in a previous study which identified common immunodominant proteins between the 2 species (Shkap et al., 1991). In addition, it is difficult to compare results of immunoprecipitation techniques used in previous experiments (Palmer et al., 1988a) with immunoblots used here. Immunoblotting may alter epitopes during the process of denaturation and electrophoretic transfer prior to interaction with antibodies. Our results do indicate however, that certain epitopes on the MSP3 protein of the FL isolate of *A. marginale* are distinctly different from those recognized by cattle infected with *A. centrale*. This could be a useful distinction since *A. centrale* is

nonpathogenic and carrier cattle are not a threat to livestock production.

In contrast, serum from sheep infected with *A. ovis* showed a strong positive reaction with MSP3 of *A. marginale* as well as a 36 kDa antigen, possibly MSP2. In previous experiments, a panel of 18 MAbs reactive to various *A. marginale* proteins failed to react with *A. ovis* infected erythrocytes (McGuire et al., 1984). The difference in results may be explained by the limited number of epitopes recognized by these MAbs. Our results confirm cross reactivity exists between at least 2 of the major antigens of *A. marginale* and serum from animals infected with *A. ovis*. These results indicate a diagnostic test using MSP3 of *A. marginale* may be able to detect *A. ovis* infected sheep. The cross reactivity between these two species should not present a problem in cattle since *A. ovis* fails to infect either intact or splenectomized animals (Jain, 1986).

The *Ehrlichia sp.* and *C. ruminantium* have been shown to be phylogenetically closely related to *Anaplasma marginale* (Dame et al., 1992; Van Vliet et al., 1992). Our results confirm cross reactive proteins do exist between these species when sera from animals infected with any of these organisms is reacted with initial body preparations from a Florida isolate of *A. marginale*. Only sera from animals infected with *E. risticii* and *E. ewingii* react with the MSP3 antigen. These species do not infect cattle. However, the results illustrate

the potential for cross reactivity between *A. marginale* and *Ehrlichia bovis*, particularly since cross-reactive antigens exists among many *Ehrlichia sp* (Holland and Ristic, 1993; Ristic and Holland, 1993; Uilenberg, 1993). This is a potential problem for serologic testing of cattle in areas where *A. marginale* and *E. bovis* coexist.

Serum from an animal infected with *C. ruminantium* does not react with an 86 kDa antigen, but does react with a protein of slightly smaller molecular mass, and an antigen which has a molecular mass of just above 30 kDa. Recently, sequence homology has been demonstrated between genes encoding immunodominant surface proteins of *A. marginale* and *C. ruminantium*. Amino acid sequence analysis revealed 33% identity and 40 similarity between the *C. ruminantium* MAP1 and the *A. marginale* MSP4 (Van Vliet, et al., 1994). Significant amino acid sequence similarity was also revealed between the 21 kDa MAP2 protein of *C. ruminantium* and the 19 kDa MSP5 protein of *A. marginale* (Mahan et al., 1994). In addition, extensive amino acid sequence homology (59% similarity; 33% identity) was conserved in multiple oligopeptide sequences throughout the MSP4 (31 kDa) protein and the MSP2 (36 kDa) protein of *A. marginale* (Palmer et al., 1994). Hence, sequence similarity was also identified between MSP2 of *A. marginale* and MAP1 of *C. ruminantium* (Palmer et al., 1994). Considering the close phylogenetic relationship between these 2 organisms, the known amino acid sequence homology between

some surface proteins, and the fact that *C. ruminantium* antiserum reacts with several *A. marginale* antigens on immunoblots, caution must be taken in the development of a diagnostic test which can distinguish between these 2 pathogens.

Sera from animals infected with *B. bovis* or *B. bigemina* showed no evidence of cross reactivity with *A. marginale* preparations. These organisms are not considered to be closely related phylogenetically (Dame et al., 1992; Van Vliet et al., 1992), nor has any similarity in antigens or DNA been detected in other experiments (Eriks et al., 1989; McGuire et al., 1984; McGuire et al., 1991; Shkap et al., 1990).

Based on our results, there is no strong evidence which would exclude MSP3 as a diagnostic test antigen based solely on the specificity of this protein. However, because there is strong cross reactivity with sera from animals infected with *Ehrlichia sp.* which do not infect cattle, the specificity of MSP3 still remains questionable, particularly in areas where *E. bovis* and *A. marginale* coexist.

Size Polymorphism of MSP3 Among Various Strains of *A. marginale*

Size polymorphism is demonstrated in the MSP3 proteins in 3 of the 4 geographic isolates studied. Marked size polymorphism has been recognized in other major surface antigens of *A. marginale*, the MSP1α protein (Oberle et al., 1988), and the MSP2 protein (Palmer et al., 1988b). Size

variation of MSP1α among geographic isolates was later explained by various numbers of tandem repeats within a single domain (Allred et al.,1990). Despite the marked size variation of MSP1α, many of the surface epitopes, including a neutralization-sensitive epitope remained conserved between strains (Allred et al., 1990; Oberle et al., 1988).

The MSP2 protein was found to be encoded by a multigene family in which substantial nucleotide sequence polymorphism exists among MSP2 copies. Variability in the expression of these genes among organisms suggests one potential mechanism for size polymorphism. This surface protein was found to be antigenically polymorphic as well (McGuire et al., 1984; Palmer et al., 1994).

Size variation has been reported in the major immunodominant surface protein of another rickettsial agent closely related to *A. marginale*, the 32 kDa MAP1 protein of *C. ruminantium* (Barbet, et al., 1994). The conservation of antigenic epitopes on the protein or the mechanism of size variation is not known.

Although the mechanism of size variation of the MSP3 protein is not known, this illustrates the organism's ability to alter important, antigenic surface proteins. The presence of a complex multigene family for MSP3 or the presence of variable numbers of tandem repeats are potential mechanisms for size polymorphism. If tandem repeats are responsible for the size polymorphism of MSP3, important antigenic epitopes

may be conserved, particularly if epitopes are contained within tandem repeats as with MSP1α. If a complex family of closely related genes is responsible for size variations, similar to the MSP2 protein of *A. marginale*, antigenic variations are more likely to exist as well. Size polymorphism in MSP3 could pose a problem to its use as a diagnostic test antigen, if it results in variation of antigenically important epitopes. This may result in poor reactivity between the MSP3 antigen of one isolate and immune sera from an animal infected with a heterologous strain.

Immune Response to MSP3

This study demonstrates variations in reactivity of immune sera from cattle infected with different geographic isolates when reacted to the FL MSP3. Multiple 86 kDa antigens are seen using immunoblots of 2-D separated preparations. Reactivity of antisera with these antigens varied depending on which geographic isolate the cattle were infected with. In a homologous reaction with anti-Fl serum 2 major areas of reactivity at pIs 6.5 and 6.2 are identified. These areas are distinctly different from the antigen recognized by the anti-MSP3 MAb and the anti-VA serum (pI 5.6). The anti-WA serum recognizes 2 entirely different 86 kDa antigens (pI 5.3 and 5.1). Only serum from SI infected cattle reacts with all the 86 kDa antigens identified with other antisera and the MAb. Although the anti-MSP3 MAb

reacted with only a single 86 kDa antigen, rabbit-Anti-MSP3 polyclonal serum reacts with antigens in areas of the pH gradient identical to those recognized by anti-FL serum, Anti-VA serum, and the ant-MSP3 MAb. This rabbit sera was made by injection of purified MSP3, isolated from an affinity column using MAb AMG75C2. The production and reactivity of this serum has previously been described (McGuire et al., 1991). Reactivity of different 86 kDa antigens with rabbit-anti-MSP3 suggests that common epitopes may exist on these antigens.

The above results indicate, similar to the MSP2 protein of *A. marginale*, not only size polymorphism, but also antigenic polymorphism exist between MSP3 antigens of different isolates. The ability of the organisms to alter this surface antigen could present a problem for use as a diagnostic test antigen, resulting in a test with low sensitivity which could not reliably detect infection in animals infected with different strains of *A. marginale*.

There are at least 3 possible explanations for the multiple 86 kDa antigens present in the FL isolate of *A. marginale*. These antigens may arise from, a) post-translational modification of a protein transcribed from single copy gene, b) several closely related genes transcribed and translated from a multigene family, or c) entirely unrelated genes. Reactivity of the rabbit-anti-MSP3 sera with multiple 86 kDa antigens suggests at least 3 of the MSP3 antigens share common epitopes. This would indicate the 86

kDa antigens are likely the result of either post-translational modification of a protein from a single copy gene, or transcription and translation of several closely related genes from a multigene family.

Previous studies have shown multigene families encoding major surface proteins of *A. marginale*. Between 7 to 10 similar gene copies encode the MSP2 gene of *A. marginale* (Palmer et al., 1994). MSP2 is a 36 kDa protein, and our results illustrate multiple MSP2 antigens using 2-D gel electrophoresis and immunoblots with an anti-MSP2 MAb (ANAF19E2). These results support the findings of the MSP2 multigene family, and suggest that transcription and translation of this family produces variations in the MSP2 protein. In addition, the MSP1β subunit of the MSP1 gene of *A. marginale* also is encoded by a partially homologous multigene family (Viseshakul et al., 1994). Genes in this family were shown to differ from each other by extensive deletions, insertions and rearrangements of sequences (Viseshakul et al., 1994).

These previous experiments, along with data presented in this study, prompted us to investigate the possibility that the MSP3 antigens could be encoded by a multigene family. If this is true, it would further demonstrate the ability of the organism to use multigene families to vary important immunogenic surface proteins. In this case, immunogenic epitopes conserved in all strains of *A. marginale* may need to

be identified if these antigens are to be useful as diagnostic test antigens or vaccine candidates.

Identification of pBluescript MSP3-12 as a Recombinant Form of an 86 kDa Antigen

Anti-MSP3 MAb AMG75C2 bound expressed protein from an MSP3 clone, pBluescript MSP3-12. The reactivity of this MAb has been previously described (McGuire et al., 1991). Because this MAb also binds to one of the 86 kDa antigens seen on 2-D gel electrophoresis (pI 5.6), we are able to identify clone MSP3-12 as a member of the MSP3 gene family. In addition, immune sera from an animal infected with a VA strain of *A. marginale*, but not a FL strain, reacts with recombinant MSP3-12. This is supportive evidence that MSP3-12 shares common epitopes with the 86 kDa antigen (pI 5.6) since VA sera strongly reacts with this antigen on 2-D immunoblots whereas immune sera from cattle infected with a FL isolate react poorly if at all.

The MSP3-12 clone contains the N-terminus of the protein as well as 633 bp upstream to the start codon. The region upstream to the open reading frame likely contains the regulatory sequences of the MSP3 gene. This helps insure the correct transcription and translation of the gene by *E. coli* since rickettsial promotors have been shown to be recognized by *E. coli* polymerases (Oaks et al., 1987). Genes containing their own promotor regions will naturally be in the correct reading frame. Anti-MSP3 MAb AMG75C2 does not bind to

proteins expressed by clones MSP3-11 or MSP3-19. The N-terminus is lacking in these clones, therefore upstream regulatory sequences of the gene are not available for *E. coli* polymerase binding. The clones are in frame with the *lacZ* gene in pBluescript, however, either they are not produced in sufficient quantity without IPTG induction, or they do not contain the epitope recognized by MAb AMG75C2.

pBluescript MSP3 is an Accurate Representation of a Genomic Copy of MSP3

Numerous artifacts can occur during the process of cloning causing disruption of the original form of a gene. In constructing a library, noncontiguous fragments of DNA may reanneal prior to ligation into the vector. In addition, many cloning artifacts may occur once the vector is transformed into the host cell. Some of these include deletions, rearrangements, or endonuclease digestion of insert DNA by the host cell. Repeats or hair-pin structures in insert DNA may not be well tolerated by some host cells such as *E. coli*, and the insert may be omitted or portions rearranged or deleted during replication. Because of these, and many other potential cloning artifacts, it must be shown that the recombinant form of MSP3 is an accurate representation of genomic MSP3. We have determined that pBluescript MSP3-12 is an accurate representation of genomic MSP3 by 1) expression of a recombinant protein which is bound by anti-MSP3 MAb and by 2) demonstration of comigrating bands of predetermined sizes

in cloned and genomic DNA when cut with restriction enzymes *Nco* I, *Bsp* M, and *Eae* I. For example, *Nci* I digestion of MSP3-12 produces a 1,176 bp fragment which hybridizes with the digoxigenin-labeled MSP3-12 probe. Digestion of genomic DNA from a FL isolate of *A. marginale* produces a fragment of identical size which also hybridizes with the MSP3-12 probe. Digestion of genomic and cloned DNA with *Eae* I and *Bsp* M produces similar results. These enzymes cut various places within MSP3-12 clone, ranging from nucleotides 12 to 2076. This range covers almost the entire 2337 nucleotide sequence of the cloned gene.

Multiple MSP3 Copies in the *A. marginale* Genome

Hybridization studies using digoxigenin-labeled MSP3-12 identified multiple copies of partially homologous MSP3 genes in the genome of FL, SI, and VA strains of *A. marginale*. Genomic DNA was digested with restriction enzymes selected to cut outside of the MSP3-12 sequence. Hybridization of the probe with a single fragment would be seen if MSP3 was encoded by a single copy gene. Multiple fragments homologous to the MSP3-12 sequence are identified. However, the exact number of copies cannot be determined because restriction sites may be polymorphic in other copies of MSP3, causing an exaggerated estimation of the number of gene copies. In addition, more than one gene copy may be present on large fragments of genomic DNA. Although unlikely, it is possible that the

uncoded region of clone MSP3-12, which was included in the probe, could cause hybridization to multiple bands not related to the MSP3 if this sequence was repeated in the genome. This region is likely to contain regulatory sequences of the MSP3 gene, but the possibility of sequence homology between this region and other unnkown multigene families of *A. marginale* does exist. To confirm this is not occurring, probes made from an internal sequence of clone MSP3-12 will need to be produced and used to probe digested, genomic, *A. marginale* DNA.

These data do suggest a copy number of at least 10 to 15 MSP3 genes in the FL and SI isolates with slightly less, 7 to 10, in the VA isolate. With the gene size of MSP3 being approximately 2.6 kbp, we estimate MSP3 occupies as much as 3.0% of the 1,250 kbp genome of *A. marginale* (Alleman et al., 1993). The exact function of this major immunodominant surface protein is unknown, however, its prevalence in the small genome of *A. marginale* suggest a need to antigenically vary this very immunogenic protein in response to stress from the host immune system. The *A. marginale* genome is estimated to contain 7 to 10 copies of MSP2, another immunodominant *A. marginale* surface protein encoded by a multigene family (Palmer et al., 1994). This gene family occupies $\geq$ 1% of the genome. The exact function of this protein is also unknown, however, immunization of cattle with affinity purified MSP2

does appear to offer at least partial protection against homologous and heterologous challenge (Palmer et al., 1988b).

Genetic polymorphism between isolates of *A. marginale* is seen when comparing isolates after digestion with the same restriction endonucleases. This is evident by variations in the length of restriction fragments which contain the MSP3 genes in each isolate. These results are consistent with previous experiments identifying restriction fragment length polymorphism between geographic isolates in ethidium bromide-stained gels (Alleman, et al., 1993). In addition, our hybridization studies indicate there are fewer MSP3 copies in the VA isolate than in the FL or SI strains. This, plus the fact that serum from animals infected with a VA isolate binds only one of the MSP3 antigens (pI 5.6), whereas serum from animals infected with Fl or SI strains react with multiple antigens, may suggest less antigenic variation of MSP3 occurs within the VA isolate.

Cloning and sequencing of several complete MSP3 genes may be required to understand the full extent of the genetic and antigenic polymorphism which exists between expressed copies of MSP3. The 3 clones made available to us contain partial gene sequences of MSP3. Although large areas of identity exist, there is also significant amino acid sequence variation. Our data suggest the amino acid sequence variation results in epitope or antigenic variation as well. This is evidenced by variable reactivity of different immune sera to

multiple MSP3 antigens. In addition, the epitope recognized by the anti-MSP3 MAb is present on only one of the 86 kDa antigens.

It has been proposed that antigenic variation plays a role in the cyclic rickettsemia and persistent infection recognized in carrier cattle infected with *A. marginale* (Kieser, et al., 1990). The level of rickettsemia varied markedly at bimonthly intervals from $<10^3$ to $>10^5$ infected erythrocytes per ml of blood (Eriks et al., 1989). The number of infected erythrocytes gradually increased over a 10 to 14 day period, then precipitously decreased (Kieser, et al., 1990). The length and consistency of the cycles suggests recurrence is due to continual antigenic variation by the organism and development of a primary immune response by the host. Further work is needed to determine if antigenic variation of MSP2 or MSP3 occurs during rickettsemia cycles in persistent carriers. This could be done by identifying copy specific epitopes on expressed MSP3 antigens and monitoring changes in parasite antigens during these cycles.

Bacteria in the genera *Borrelia* and *Neisseria* have been shown to use multigene families to vary important surface antigens and aid in the evasion of the host immune system (Meyer et al., 1990; Barbour, 1990). The genome of *Borrelia hermsii*, the causative agent of relapsing fever, contains a large repertoire of genes encoding variable major proteins (Vmps) (Barbour, 1990; Barbour, 1991). Multiple silent and

active copies of the *vmp* genes are located on linear, extrachromosomal, DNA plasmids. The promotor and active copy of the genes are located at telomeric ends of the linear plasmids. Switching of a silent copy to the active locus just downstream from the promotor causes expression of an antigenically different Vmp and conversion of the organism from one serotype to another. Antigenic variation allows *Borrelia hermsii* to evade the host immune system and avoid complete clearance from the blood stream. This causes a persistent illness with a cyclic rise and fall in body temperature every 4 to 7 days (Barbour, 1991).

Neisseria gonorrhoeae uses multigene families to antigenically vary 2 important adherence ligands, the pili and outer membrane opacity proteins (Opa) (Meyer et al., 1990; Sparling et al., 1990). Variations in pili are accomplished by multiple, silent, incomplete copies (over 20) of *pil* genes termed minicassettes (Sparling et al., 1990). Insertion of one of these incomplete copies into an expression site can result in the expression of an antigenically different pilus. In contrast, 10 - 12 complete *opa* genes are present in *Neisseria gonorrhoeae*, and more than one may be expressed simultaneously (Meyer et al., 1990). Expression of individual *opa* genes is dependent on a repetitive sequence, the coding repeat, which encodes the Opa signal peptide. The number of 5-mer repeats present determines if the *opa* gene is translationally in frame. Genes in which 5-mer repeats occur

in multiples of 3 are expressed while those with any other multiples are not produced (Meyer et al., 1990). Variations in the number of 5-mer repeats occurs frequently during DNA replication. Antigenic variation allows *N. gonorrhoeae* to persist in the host unless appropriate antibiotic therapy is instituted, and produce repeated infections in the same host (Sparling et al., 1990)

The exact mechanism by which *A. marginale* uses multigene families is not known. However, like *Borrelia hermsii* and *Neisseria gonorrhoeae*, *A. marginale* does appear to evade the host immune system and avoid complete clearance. In addition, as in relapsing fever, fluctuating parasitemia is observed in cycles consistent with the appearance of antigenic variants in response to immune pressure from the host (Krieser et al., 1990). We hypothesize *A. marginale* could use mechanisms similar to those seen in *B. hermsii* and *N. gonorrhoeae* to vary important antigenic surface proteins such as MSP2 and MSP3 in an effort to avoid immune clearance.

Distribution of MSP3 Copies in the *A. marginale* Genome

Previously, we have shown that by *Sfi* I digestion and separation of large fragments using CHEF electrophoresis, we can separate the entire genome of *A. marginale* (FL) into 14 fragments ranging in size from 160 to 14 kbp (Alleman et al., 1993). The MSP3-12 probe hybridizes to 6 of these fragments, as well as multiple fragments in *Not* I and *Sfi* I digests of

other isolates. However, judging from the intensities of the bands, it appears most of the copies in the FL isolate are located on two large *Sfi* I fragments approximately 160 and 130 kbp. The smaller band has been shown to contain a doublet of comigrating fragments (Alleman, et al., 1993). Therefore, these two hybridizing bands could represent as much as 25% to 37% of the genome size. Because this is such a large area of the genome, conclusions regarding the proximity of the genes are difficult to make. The *Not* I digest of the FL isolate, and the *Not* I and *Sfi* I digests of the other isolates indicates a more even distribution of copy numbers throughout the genome.

This suggests MSP3 copies are widely distributed throughout the *A. marginale* genome, similar to the pattern seen with the MSP2 multigene family (Palmer et al., 1994). These copies are likely not the result of simple duplication of a single MSP3 gene since the copies are not present in tandem along a single stretch of DNA. This assumption is supported by the partial gene sequences available for 3 of the MSP3 genes. This would indicate that any coordinated regulation of MSP3 copies would involve *trans*-regulation.

Summary and Conclusions

We have determined that the MSP3 antigen of *A. marginale* is of questionable specificity as a diagnostic test antigen with potential for cross reactivity with sera from animals

infected with *Ehrlichia sp.* We have also shown that this antigen is not conserved among various strains of *A. marginale*, and that antigenic variation likely exists between isolates since immune sera from cattle infected with different strains reacted differently to MSP3.

It has now been shown that 2 major surface antigens of *A. marginale*, MPS2 & MSP3, are actually composed of a family of related proteins. Using an MSP3 clone as a probe in hybridization studies we concluded the multiple MSP3 antigens are the result of a complex, multigene family of partially homologous genes, similar to the MSP2 protein. Although we cannot state definitively, we estimate that a relatively large portion of this rickettsial agent's small genome (up to 4%) is occupied by these 2 gene families. We hypothesize the organism uses these multigene families to antigenically vary these major immunogenic surface proteins.

The cross reactivity of this protein with sera from animals infected with *Ehrlichia sp.*, the size polymorphism of MSP3 between different geographic isolates, the multiple 86 kDa antigens recognized by various antisera, and the presence of a multigene family encoding these antigens indicate that in its native form, a single recombinant MSP3 would not be a suitable candidate for use as a diagnostic test antigen. In order to be used as a test antigen, it may be necessary to define and obtain expressed copies of the MSP3 gene. The potential for using multiple recombinant MSP3 antigens

produced by the expressed copies could then be evaluated. Alternatively, conserved epitopes on these genes could be identified and recombinant or synthetic peptides derived from gene sequences could be tested with immune cattle sera to determine their reactivity. However, considering the apparent ability of *A. marginale* to antigenically vary this protein, we feel these attempts may not be practical, particularly since another surface antigen (MSP5) has shown some promise for use as a diagnostic test antigen (Ndung'u et al., 1995). This 19 kDa protein is encoded by a single copy gene and appears to be conserved between all recognized *Anaplasma* species (Visser et al., 1992).

Even though MSP3 may not be an ideal test antigen, the results of these experiments may provide valuable information regarding the use of this antigen in a subunit vaccine. It is not known if response to any of the MSP3 antigens provides protective immunity to cattle. However, the demonstration of multiple 86 kDa proteins, and antigenic variation between these proteins, would indicate that multiple expressed copies of MSP3 may need to be evaluated for potential use in vaccine trials. The need for the organism to antigenically vary this surface protein suggests it serves an important function for survival in the host. Immune response to an area conserved between all expressed copies may prove beneficial in neutralizing infectivity.

The information presented in this study may also be valuable in studying antigenic variation of *A. marginale* in persistently infected, carrier cattle. Copy-specific epitopes on an MSP3 molecule could be defined, and variations in these epitopes could be monitored in cyclic rickettsemias of carrier cattle. This would provide much needed information regarding the mechanisms by which this organism, and possibly other rickettsial agents, evade the host immune system. Basic information regarding the means by which organisms adapt to their host helps establish better ways to diagnose, prevent, and eventually eradicate these diseases.

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BIOGRAPHICAL SKETCH

A. Rick Alleman was born in New Orleans, LA, on August 23, 1954. He is married to Mary Alleman, and they have 2 children, Arthur Rick Jr. and Grace Elizabeth.

Rick completed his undergraduate studies at the University of New Orleans in 1976. He then attended Louisiana State University, School of Veterinary Medicine in Baton Rouge where he obtained his Doctor of Veterinary Medicine degree in 1980. At this time he practiced small animal medicine in New Orleans, LA, with his partner Mariano Guas. In 1986 Rick became board certified by the American Board of Veterinary Practitioners (ABVP) as a specialist in companion animal medicine.

In 1989 he left private practice and, under the guidance of Drs. Rose Raskin and John Harvey, joined the University of Florida College of Veterinary Medicine to do a residency in clinical pathology. In 1992 he completed his residency training and was board certified by the American College of Veterinary Pathologists (ACVP), specialty Clinical Pathology.

In 1990, under the guidance of Dr. Anthony Barbet, Rick entered into a Ph.D. graduate program in the Department of Pathobiology. During his doctoral research program he developed a keen interest in the study of rickettsial agents

at the molecular level. He plans to pursue a career in academia where he will continue his laboratory investigation of rickettsial agents as well as provide instructional services to students and clinical services to the teaching hospital.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John B. Dame, Chair
Associate Professor of
Veterinary Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Anthony F. Barbet
Professor of Veterinary
Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John W. Harvey
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Veterinary Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1995

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